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13. ABSTRACT ***(Maximum 200 Words)***

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. In this study we report that HA increases mitogenesis in both normal and malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis. We have also shown that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. HA crystals enhance the production of a variety of MMPs in normal and breast cancer cell lines alone or in co-culture. In addition our results suggest that exposure to HA crystals can cause a significant increase in PGE_2 by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells. Immunohistochemical staining of breast biopsies however did not show enhanced expression of MMP-1 or MMP-9 localised around the calcifications. COX-2 staining was occasionally upregulated in the proximity of a calcification and this finding warrants further study. Characterisation of the calcifications in the biopsies is necessary to help explain these finding. The data generated with the aid of this award supports our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

14. SUBJECT TERMS
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Title: Biological effects of calcium hydroxyapatite crystals in breast cancer.
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Introduction

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Analysis of these microcalcifications by electron microscopy, microprobe analysis and X-ray diffraction has shown that in breast tissue two principle types of calcifications can be distinguished according to their structure and chemical composition (Harris, Morrow et al. 1993). Type I microcalcifications are composed of calcium oxalate in the form of weddellite crystals and type II microcalcifications consist of calcium phosphates in the crystalline form of hydroxyapatite (HA), ($Ca_{10}(PO_4)_5(OH)_2$), which is also the basic calcium phosphate found in mature bones and teeth. There is evidence that calcium phosphate and oxalate tend to be associated with different kinds of breast lesions (Busing, Keppler et al. 1981). The presence of oxalate-type microcalcification appears to be a reliable criterion in favour of the benign nature of the lesion or, at most, of an *in situ* lobular carcinoma and are rarely associated with malignancy (Radi 1989; Going, Anderson et al. 1990). In contrast, the calcifications associated with malignant breast lesions are generally formed by hydroxyapatite (Frappart, Boudeulle et al. 1984).

Although their diagnostic value is of great importance radiographically, the genesis of breast calcifications is unclear. The occurrence of microcalcifications has not been shown to be significantly associated with age or primary tumour size. However, several studies have shown that survival of patients with mammographic microcalcification was significantly shorter than those without (Tsuchiya, Kanno et al. 1996),(Holme, Reis et al. 1993). A recent study by Tabar *et al.* also showed that the relative hazard of death from breast cancer was five times higher for tumours with casting-type calcifications than that for circular lesions with no calcifications (Tabar, Chen et al. 2000). There have been numerous histological ultrastructure studies of HA deposits in breast carcinomas. However, despite their potent biological effects in other systems and their association with poorer survival in breast cancer patients, to date there have been no investigations of their potential role in the growth and progression of breast tumours. In the present study, we investigated the pathogenic potential of calcium HA crystals in human breast cancer cell lines and human fibroblasts by studying their ability to induce mitogenesis, and upregulate PGE_2 and MMP production.

This final report covers research for the period August 1st, 1997 to July 31st, 2001. An extension of the award period was awarded due to the principal investigators change of institutions in July 1998. In the extension period we completed Task 4 of the statement of work; Immunohistochemical study of MMP family in surgical specimens from breast cancer patients with microcalcifications.

Body

- The first two specific aims of the proposal as outlined in the technical objective have been addressed, namely 1) study of the mitogenic response of cells in culture to HA crystals, and 2) investigation and characterisation of the induction of

MMPs by HA crystals in cell culture. The results of which are summarised in two manuscripts, both attached in the appendix.

- Manuscript published: 'Calcium Hydroxyapatite Promotes Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines' (2001), **Maria P. Morgan**, Michelle Cooke, Pamela A. Christopherson, Pamela R. Westfall, and Geraldine M. McCarthy, Molecular Carcinogenesis, 32: 111-117. See appendix for copy of manuscript.
- Manuscript in preparation: 'Molecular Mechanism of Calcium Hydroxyapatite induced Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines', Michelle M. Cooke, Geraldine M. McCarthy and **Maria P. Morgan.** See appendix for copy of manuscript.

- Additional data which is not included in any manuscript to date is attached in the appendix, figures 1-6.

Technical Objective 3, Task 4

The third technical objective involved analysing the pattern of HA-associated MMP and COX-2 protein expression in surgical specimens. A collaboration was established with an Irish pathologist Dr. Maria Kennedy, based at Breast Check, The National Breast Screening Programme, The Mater Hospital, Dublin, Ireland. Ethical approval was granted for the study and specimens collected. We studied the expression and localisation of 3 genes, MMP-1, MMP-9 and COX-2 in human breast biopsies containing calcifications. We choose to look at MMP-9 and COX-2 because we had shown that both genes were upregulated by HA crystals in breast cancer cells in our *in vitro* studies. MMP-1 was also chosen as in previous studies by our group it has been shown to be upregulated in human fibroblasts by HA crystals.

All biopsies examined were from ductal carcinoma in situ (DCIS), since these are the tumours most frequently associated with microcalcifications. A total of fifteen sections were analysed by immunohistochemistry (IHC). This was a smaller sample size than the original number suggested in the proposal however, there were many factors which made a larger study size infeasible. Obtaining biopsies was fraught with difficulty since;

a) we required biopsies which contained microcalcification, Faxitron x-ray of each biopsy was used to confirm the presence of microcalcification and samples with a characteristic HA pattern (as assessed by a pathologist) were processed further.

b) each microcalcification had to be confirmed as hydroxyapatite (hydroxyapatite stains purple with haemotoxylin, oxalate does not stain).

c) limited material from each suitable sample was available since it was required for the patients diagnosis and records, and many of these biopsies were by definition very small, taken from small, unpalpable lesions.

d) the Irish national breast screening programme, Breast Check, was only established in 1998 and no archive material was available to us and, since its initiation pathology staff on the programme have been overwhelmed with the work load, but tried to facilitate our study as much as possible.

e) Following recent media reports in Ireland and the UK with regard to tissue and organ retention for research purposes in hospitals, Irish patients are more reluctant to participate in research studies where tissues or parts of tissues are

involved. This delayed our application for ethical approval and subsequent acquisition of samples.

Sections were cut from formalin-fixed, paraffin-embedded tissue blocks of breast biopsies. Prior to probing with the antibody, the sections were dewaxed and rehydrated. Vector ABC staining kit was used for the immunohistochemistry protocol. We encountered some difficulty finding a source for antibodies to MMP-1 and MMP-9 which where suitable for use on paraffin-embedded tissue. Initial optimisation of antibody concentrations and antigen retrieval procedures was required for all three antibodies. An antigen retrieval step consisting of boiling the sections in citrate buffer for 6 minutes was found to be optimum for MMP-1 and MMP-9. Figure 1 shows the effect of an antigen retrieval step in improving specific staining. No antigen retrieval step was necessary for COX-2 immunohistochemistry. The primary antibodies were used at the following concentrations; MMP-1 (1μg/ml), MMP-9 (1μg/ml) and COX-2 (1μg/ml).

The extracellular matrix (ECM), composed of structural components such as proteoglycans and glycoproteins, forms the external scaffold within which normal and tumour cells reside. Excessive breakdown of ECM is one of the hallmarks of cancer and is an important component of the process of tumour progression. In order for tissues to change their form or function, the ECM must be degraded and the tissue remodelled. Although, in general there are higher levels and greater numbers of MMPs expressed in highly invasive and metastatic tumours, MMP levels can be elevated even in the early stages of tumour progression. Table 1 summarises the general staining pattern observed for COX-2, MMP-1 and MMP-9 protein expression in the breast biopsies. Figures 2-4 show examples of the staining patterns observed and shows the prominent features of the breast biopsies. In general a similar staining pattern was seen for MMP-1 and MMP-9. This consisted of intense staining of epithelial cells lining the ducts with faint staining of stromal areas which is consistent with published reports of IHC staining for MMP-1 and –9 [8]. When human tumour tissues are examined closely for MMP expression patterns, it is not only the tumour that over expresses MMP family members, but also the surrounding, normal stromal tissue. The tumour stroma is made up primarily of fibroblasts, infiltrating immune cells and endothelial cells. Part of this over expression in the stromal tissue is thought to be due to the fact that the host tissue reacts as though the tumour is a wound: triggering a wound healing response that involves MMPs. The tumour cells induce MMP expression either through direct contact or by secreting soluble factors that trigger MMP expression in the stroma. The interaction between cell integrins and matrix is now known to trigger signal transduction cascades that broadly affect a variety of cellular functions, including cell survival, proliferation, differentiation and migration.

Numerous studies have demonstrated a role for COX in breast cancer. Epidemiologic studies have demonstrated that the use of nonsteroidal antiinflammatory drugs (NSAIDs) can reduce the risk of breast cancer. Published reports of IHC staining of specimens that expressed COX-2 revealed that COX-2 was localised primarily in the tumour cells but also appeared in stromal cells [9]. In our study we found that staining for COX-2 was localised around the ducts as shown in figure 2,3,4, with some faint staining of stromal areas also. Table 2 shows the relative intensity of staining of either epithelial cells or stromal tissue in each section assessed by eye and scored accordingly for MMP-1, -9 and COX-2. In table 3, we focused on the IHC staining of areas in the immediate vicinity to a microcalcification. The

staining of areas of specimens surrounding calcifications showed a pattern of staining for MMP-1 and MMP-9 that did not differ from that noted for areas containing no calcifications (figures 5&6). For COX-2 the same appeared to be true (figure7), however there were areas surrounding a calcification in 3 different biopsies, which stained intensely for COX-2 (figure 8). The staining did not appear to be artifactual due to the coloured substrate, DAB, being trapped in the particular areas around the calcification as multiple washings were performed, but this could be a possibility. There are no published reports of IHC studies in breast tissue where localisation of the staining (for any protein) was examined relative to the presence of microcalcification. In this study, with our limited sample size, no significant correlation was seen between the presence of microcalcification and the localisation of MMP-1, MMP-9 or COX-2 protein expression in breast biopsies. This study is however being continued (by Maria Morgan PhD who was awarded a US Army post doctoral fellowship), as more samples become available to try to unravel the discrepancy between the *in vitro* and *in vivo* results. Future work will include temporal studies to look at the long term effects of HA *in vitro*.

In collaboration with Dr. Rob Lewis, Daresbury Synchrotron Laboratory, Warrington, UK and Dr. Keith Rodgers, Reader in Crystallography, Cranfield University, Wiltshire, UK, we are continuing to attempt to characterise the calcifications present in breast core cut biopsies using a synchrotron source.

Brief Summary of Entire Project:

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. In this study we report that HA increases mitogenesis in both normal and malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis. We have also shown that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. HA crystals enhance the production of a variety of MMPs in normal and breast cancer cell lines alone or in co-culture. In addition our results suggest that exposure to HA crystals can cause a significant increase in PGE_2 by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells. Immunohistochemical staining of breast biopsies however did not show enhanced expression of MMP-1 or MMP-9 localised around the calcifications. COX-2 staining was occasionally upregulated in the proximity of a calcification and this finding warrants further study. Characterisation of the calcifications in the biopsies is necessary to help explain these finding. The data generated with the aid of this award supports our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

Key Research Accomplishments

Technical Objective 1.

- We found that HA crystals increase mitogenesis in two cancer cell lines (MCF-7 & Hs578T) and one normal human mammary cell line (HMEC).
- Latex beads, particles of a similar size to the crystals had no effect on mitogenesis.
- We confirmed that the increase in mitogenesis shown by increased DNA synthesis /thymidine incorporation can also be seen in an increase in actual cell numbers.
- We further characterized the mitogenic response to HA in three cell lines and show that the increase in mitogenesis is dose dependent.
- The effect of bafylomycin A_1 (BAF), (a specific inhibitor of the vacuolar type proton pump ATPase responsible for acidification in vacuolar compartments, inhibits intracellular crystal dissolution) on HA induced mitogenesis was examined. We confirmed that phagocytosis and intracellular crystal dissolution is required for HA-induced mitogenesis.

Technical Objective 2.

- We found HA induced expression of members of the matrix metalloproteinase family, including MMP-9 and MMP-13.
- RT-PCR with primers specific for COX-2 confirmed a dose dependent induction of COX-2 by HA in the Hs578T breast cancer cell and a time course showed maximal induction 4-8 hours post stimulation.
- Our results suggest that exposure to HA crystals can cause a significant increase in PGE_2 possibly by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells.
- Calcium hydroxyapatite crystals were found to induce an anti-angiogenic effect in an *in vitro* angiogenesis assay.
- Calcium hydroxyapatite crystals cause increased production of prostaglandin E_2 by induction of both cyclooxygenase–1 and cyclooxygenase-2 in human fibroblasts.
- Calcium hydroxyapatite causes induction of the cytokine IL-1β mRNA in HFF.

Technical Objective 3.

- In general a similar immunohistochemical staining pattern was seen for MMP-1 and MMP-9, consisting of intense staining of epithelial cells lining the ducts with very little staining of stromal areas. Less intense staining for COX-2 was also localised around the ducts with some faint staining of stromal areas also.
- Areas of specimens surrounding calcifications showed a pattern of staining for MMP-1 and MMP-9 that did not differ from that noted for areas containing no calcifications.
- For COX-2 the same appeared to be true, however rarely there was an area surrounding a calcification that stained intensely for COX-2.

Reportable Outcomes

Research

- Abstract submitted to Sixth Annual Biomedical Research Symposium, St. Vincent's Hospital, Dublin, Ireland, November 1999. 'Novel pathogenic

mechanisms in breast cancer; role of calcium hydroxyapatite crystals'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM. Winner of 'Best Poster Presentation'.

- Abstract submitted to Irish Association for Cancer Research Annual Meeting, Galway, Ireland, April 2000. 'Biological effects of calcium hydroxyapatite in breast cancer cell lines'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM, Irish Journal of Medical Science (in press).
- Abstract submitted to Royal College of Surgeons in Ireland Research Day, April 2000, 'Calcium hydroxyapatite in breast cancer'; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM.
- Abstract submitted to the DoD Era of Hope Breast Cancer Research Program Meeting, Atlanta, USA, June 2000, entitled 'Novel pathogenic mechanisms in breast cancer; role of calcium hydroxyapatite crystals'; McCarthy GM, Westfall, PR, Christopherson, PA, and Morgan, M.
- Manuscript entitled 'Calcium Hydroxyapatite Promotes Mitogenesis and Induces Prostaglandin E_2 and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines', Maria P. Morgan, Pamela A. Christopherson, Pamela R. Westfall, Lynn M. Matrisian and Geraldine M. McCarthy, Molecular Carcinogenesis, (in press).

Career Development

- Maria Morgan PhD, a post-doctoral fellow was awarded a post-doctoral research fellowship from the Department of Defence Breast Cancer Research and Material Command's office of Congressionally Directed Medical Research Program for proposal entitled 'Molecular mechanisms of calcium hydroxyapatite crystal-induced mitogenesis in breast cancer' (BC990714), covering period: July 2000 – July 2003. The application was based on preliminary work supported by this award.
- Maria Morgan PhD, was also subsequently awarded an Irish Health Research Board post-doctoral research fellowship (PD-06/99) but did not accept it.
- Geraldine McCarthy hosted a mini-symposium on 'Synchrotron Radiation and Calcification in Breast Cancer', April 2000, sponsored by Bristol-Myers Squibb. Speakers included Dr. Rob Lewis Daresbury Synchrotron Laboratory, Warrington, UK, Dr. Keith Rodgers, Reader in Crystallography, Cranfield University, Wiltshire, UK, and numerous pathologists from both the UK and Ireland.
- Geraldine McCarthy (Principle Investigator) was awarded a grant from The Wellcome Trust for a project entitled 'Biological effects of calcium-containing crystals', covering period August 2000-August 2003. The application was based on preliminary work supported by this award.
- Proposal submitted to Bristol-Myers Squibb Foundation, Better Health for Women Foundation initiative in July 2000 based on results generated with the support of this award.
- Attended the Mater International Breast Cancer Meeting, Mater Misericordiae Hospital, Dublin, Ireland, February, 2001.
- Geraldine McMarthy and Maria Morgan in collaboration with Dr. Rob Lewis Daresbury Synchrotron Laboratory, Warrington, UK and Dr. Keith Rodgers, Reader in Crystallography, Cranfield University, Wiltshire, UK, had one days

beam-time at the Daresbury Synchrotron Laboratory characterising the calcification present in breast core cut biopsies, May 2001.

- March 2001, Maria Morgan was awarded a travel fellowship from Enterprise Ireland, International Collaboration Programme 2001 based on preliminary work supported by this award.
- July 2001, Maria Morgan attended the First International Symposium on 'Recent Advances and Future Directions in Breast and Ovarian Cancer' Dublin, Ireland.

Conclusions

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction and increased PGE_2 synthesis may be part of a programme of gene expression designed for malignant growth. These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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Tsuchiya, A., M. Kanno, et al. (1996). "The evaluation of mammographic microcalcification as biological malignancy in breast cancer." Fukushima J Med Sci **42**(1-2): 17-22.

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- Calcium Hydroxyapatite Promotes Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines' (2001), **Maria P. Morgan**, Michelle Cooke, Pamela A. Christopherson, Pamela R. Westfall, and Geraldine M. McCarthy, Molecular Carcinogenesis, 32: 111-117.

- Inflammatory microcrystals induce murine macrophage survival and DNA synthesis; Hamilton, J.A., McCarthy, G, and Whitty, G, Arthritis Res, 2001, **3**, 242-246.

- Manuscript in preparation: 'Molecular Mechanism of Calcium Hydroxyapatite induced Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines', Michelle M. Cooke, Geraldine M. McCarthy and Maria P. Morgan.

MEETING ABSTRACTS

- Novel pathogenic mechanisms in breast cancer; role of calcium hydroxyapatite crystals; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM. Sixth Annual Biomedical Research Symposium, St. Vincent's Hospital, Dublin, Ireland, November 1999. Winner of 'Best Poster Presentation'.
- Biological effects of calcium hydroxyapatite in breast cancer cell lines; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM, Irish Association for Cancer Research Annual Meeting, Galway, Ireland, April 2000. Irish Journal of Medical Science (in press).
- Calcium hydroxyapatite in breast cancer; Morgan, M, Westfall, PR, Christopherson, PA, and McCarthy GM. Royal College of Surgeons in Ireland Research Day, April 2000.
- Novel pathogenic mechanisms in breast cancer; role of calcium hydroxyapatite crystals; McCarthy GM, Westfall, PR, Christopherson, PA, and Morgan, M. DoD Era of Hope Breast Cancer Research Program Meeting, Atlanta, USA, June 2000.

PERSONNEL

Personnel receiving pay from the research effort:

- Geraldine M. McCarthy (principle investigator)
- Pamela Westfall (research assistant)
- Maria P. Morgan (post doctoral research fellow)

APPENDICES

Includes figures 1-8, tables 1-3 and 3 manuscripts.

Table 1. Summary of staining patterns observed for COX-2, MMP-1 and MMP-9 protein expression in breast biopsies.

Protein	General Staining Pattern Observed
COX-2	Staining was localised around the ducts with some faint staining of stromal areas also.
MMP-1	Intense staining of epithelial cells lining the ducts with no staining of stromal areas.
MMP-9	Strong staining of epithelial cells lining the ducts with very little staining of stromal areas.

Table 2. Staining intensities of COX-2, MMP-1 and MMP-9 in breast biopsies containing calcifications.

Specimen No.	Intensity of COX-2 staining		Intensity of MMP-1 staining		Intensity of MMP-9 staining	
	Epithelial cells	Stromal tissue	Epithelial cells	Stromal tissue	Epithelial cells	Stromal tissue
24/99 A3	+	+	++	–	NE	NE
24/99 A6	+++	++	+++	–	+	+
544/99 A8	-	++	+	–	+	+
544/99 A5	-	+	+++	–	+++	–
1002/99 A6	NE	NE	++	–	NE	NE
1002/99 A2	+	++		–	++	+
1996/99 B3	+	+	++	–	++	–
2495/99 A7	–	+	++	–	+	–
4149/99 A5	+	–	++	–	NE	NE
5484/99 B2	+	+	+++	–	+	+
5757/99 A3	+	+	–	–	+	+
5758/99 A7	+	++	+++	–	++	+
4250/99 A6	+	+	+++	–	+	+
5758/99 A6	+	++	+++	–	+	–
6164/99 A11	+	+	+	–	–	-

The relative intensity of staining of either epithelial cells lining ducts or stromal tissue in each section was assessed by eye and scored accordingly. – indicates no staining observed; + indicates low level of staining; ++ indicates moderate staining; +++ indicates a high level of intense staining. NE indicates specimen not examined due to lack of material.

Table 3. Summary of immunohistochemical staining localised specifically around microcalcification.

Specimen No.	COX-2 Staining	MMP-9 Staining	MMP-1 Staining
24/99 A3	N	N	N
24/99 A6	N	N	N
544/99 A8	N	N	N
544/99 A5	Y	N	N
1002/99 A6	N	N	N
1002/99 A2	Y	N	N
1996/99 A3	N	N	N
2495/99 A7	Y	N	N
4149/99 A5	N	N	N
5484/99 B2	N	N	N
5757/99 A3	N	N	N
5758/99 A7	N	N	N
4250/99 A6	N	N	N
5758/99 A6	N	N	N
6164/99 A11	N	N	N

Y indicates increased staining was localised around microcalcification; N indicates no increased staining localised around microcalcification.

Figure Legends

Fig. 1 Effect of antigen retrieval step on MMP-1 immunohistochemical staining. Staining is more specific when sections were boiled for 6 minutes in citrate buffer.

Fig. 2 Localisation of MMP-1 (A), MMP-9 (B) and COX-2 (C) expression in breast biopsy section 5758/99 A7.

Fig. 3 Localisation of MMP-1 (A), MMP-9 (B) and COX-2 (C) expression in breast biopsy section 4149/99 A5.

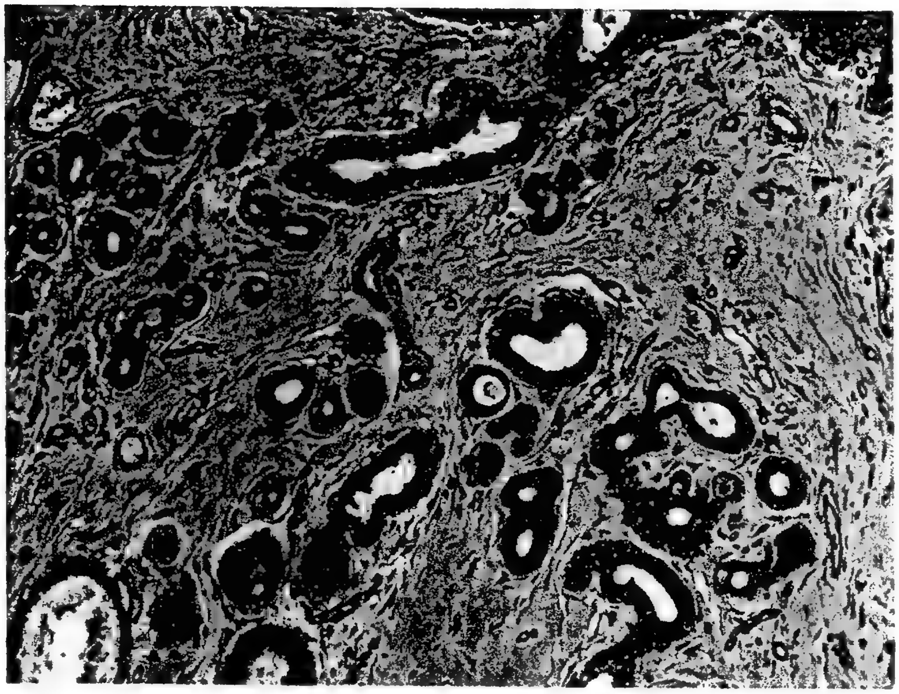
Fig. 4 Localisation of MMP-1 (A), MMP-9 (B) and COX-2 (C) expression in breast biopsy section 2495/99 A7.

Fig. 5 MMP-1 staining of 24-99 A6, (A) shows normal MMP-1 staining of ducts. (B) arrows show calcification stained purple with haematoxylin.

Fig. 6 MMP-9 staining of 24-99 A6, (A) shows normal MMP-1 staining of ducts. (B) arrows show calcification stained purple with haematoxylin.

Fig. 7 COX-2 staining of 2495-99 showing no staining localised around calcification.

Fig. 8 COX-2 staining of 5758-99 A7 , showing localisation of COX -2 around the calcification. (A) at 10X magnification and, (B) at 40X magnification.



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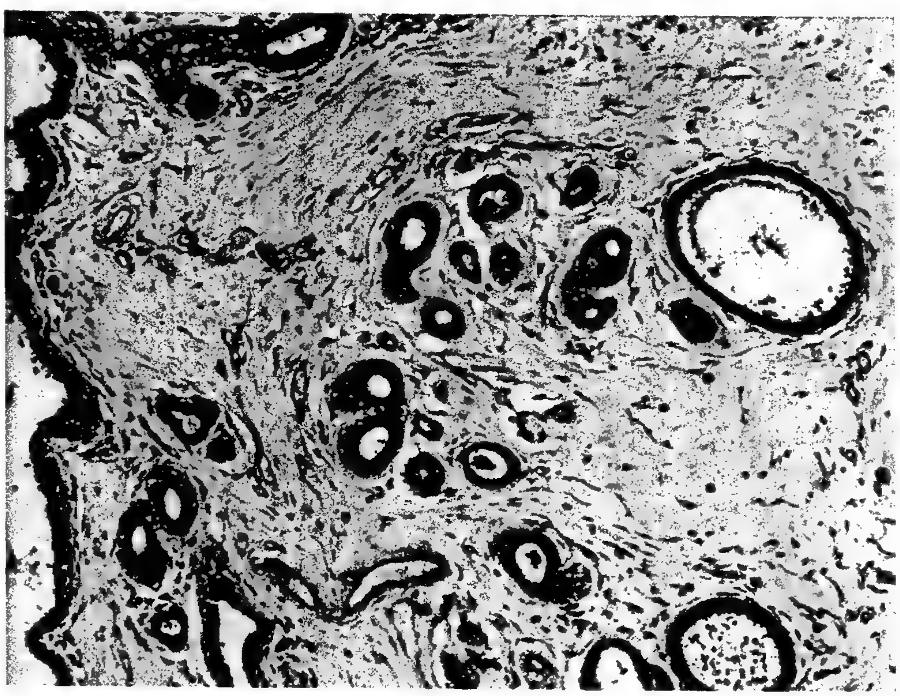
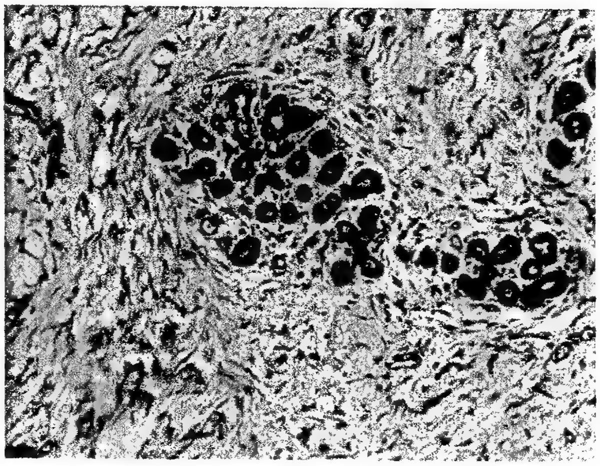


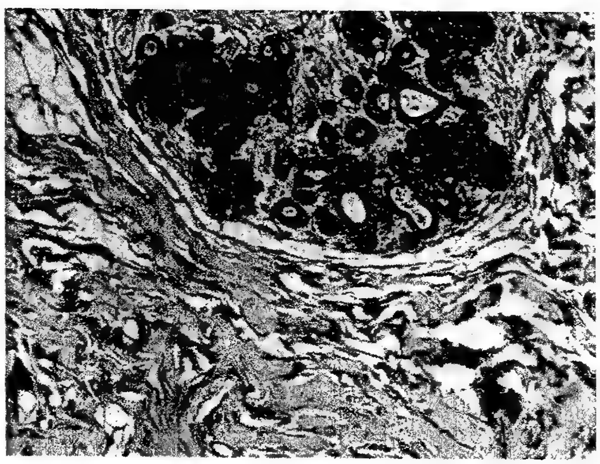
6 min antigen retrieval

Figure 1. Section 5758/99 A7 stained for MMP-1, with and without antigen retrival.

5758/99 A7



MMP-1



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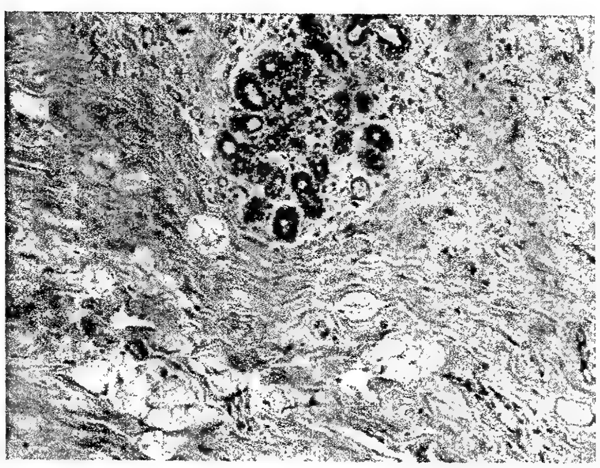
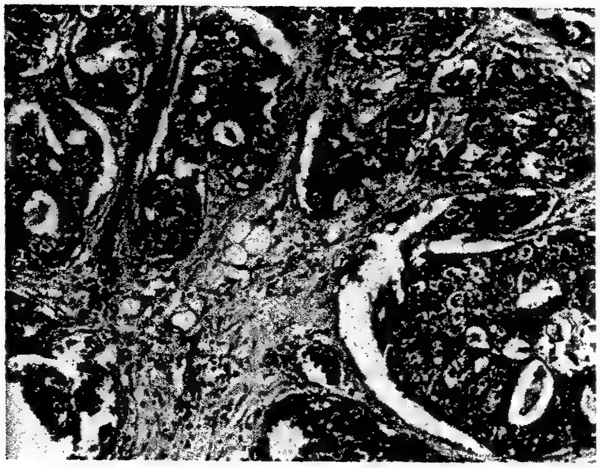


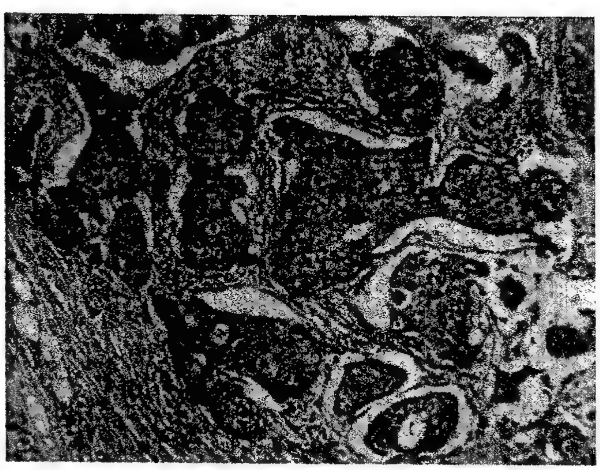
COX-2

Figure 2



MMP-1

MMP-9

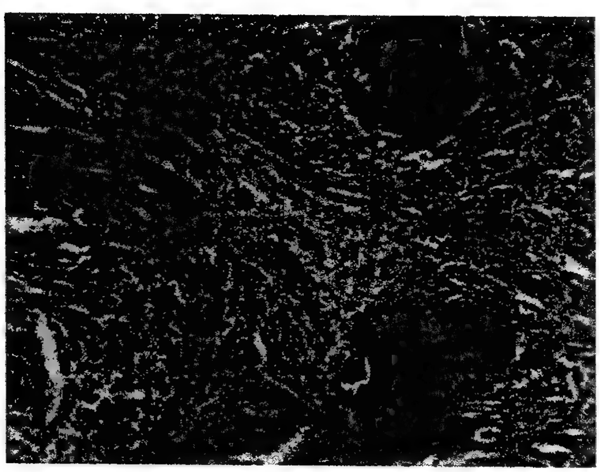


COX-2

Figure 3

MMP-1

MMP-9



COX-2

Figure 4

A

B

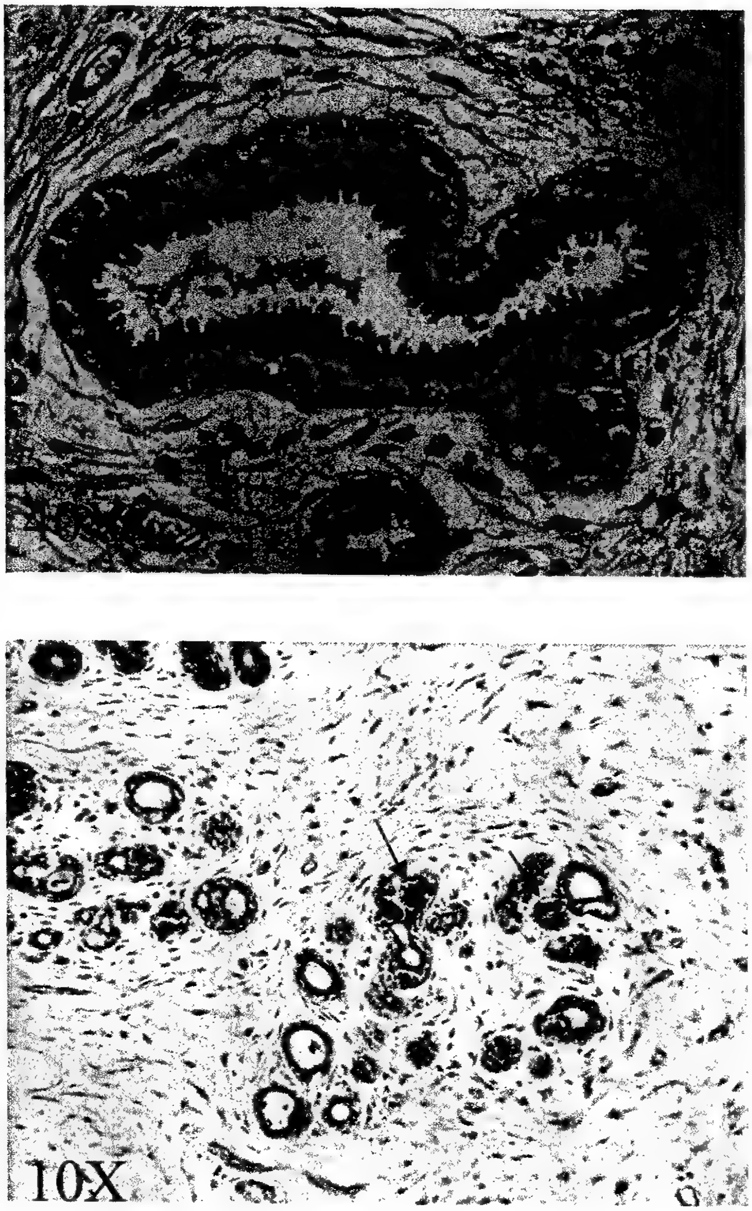
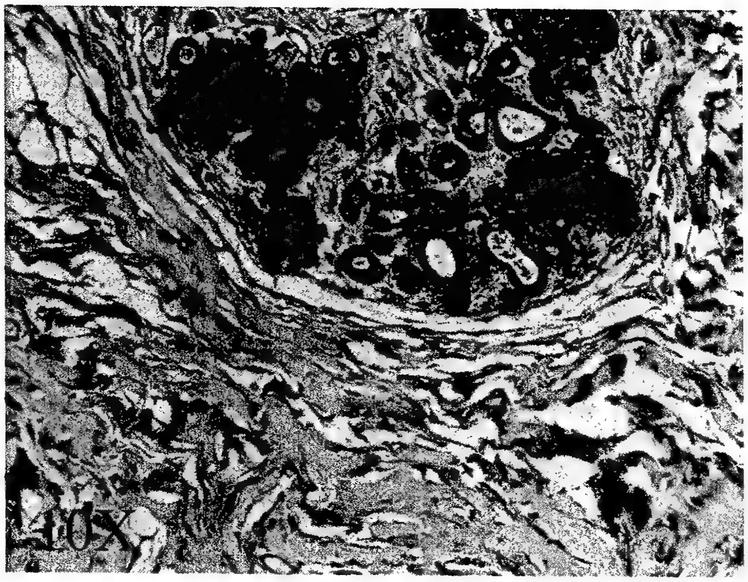


Figure 5. MMP-1 staining of 24-99-A6, (A) shows normal MMP-1 staining of ducts. (B) arrows show calcification stained purple with haemotoxylin.

A



B

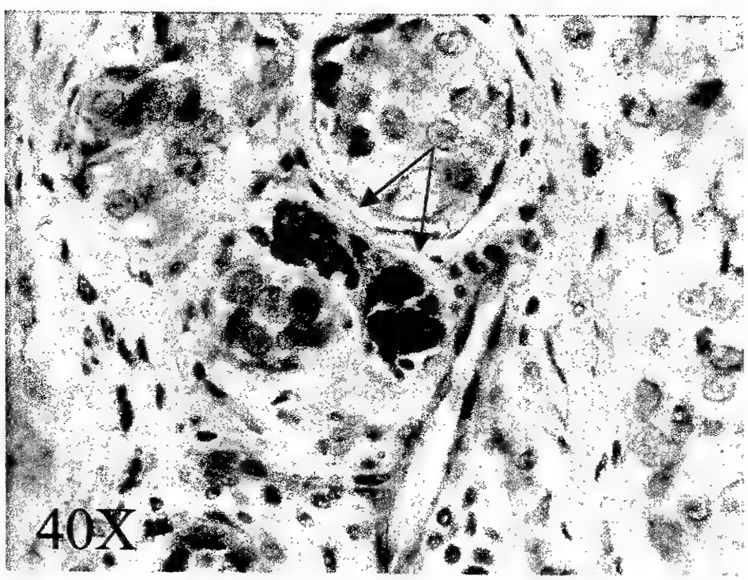


Figure 6. MMP-9 Staining of 24-99 (A) shows normal MMP-9 staining of ducts. (B) arrows show calcification stained purple with haemotoxylin.

A

Figure 7. COX-2 staining of 2495-99showing no staining around calcification.

A

B

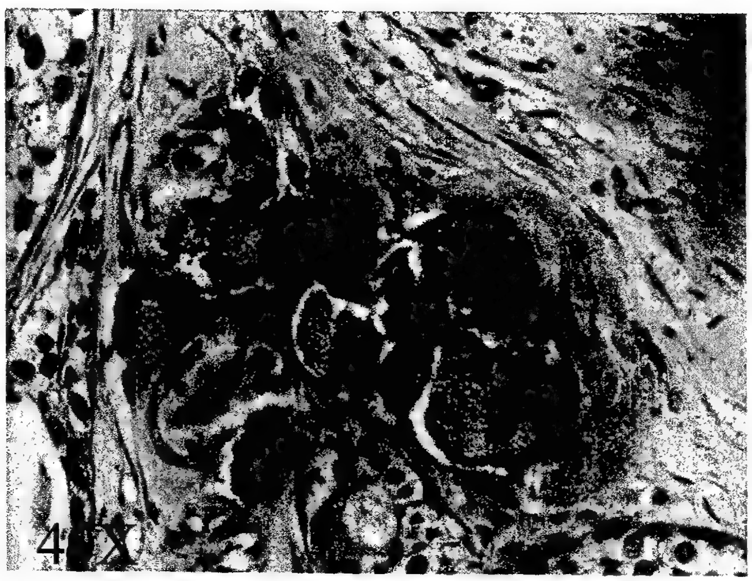


Figure 8. COX-2 staining of 5758-99 A7 , showing localisation of COX -2 around the calcification. (A) at 10X magnification and (B) at 40X magnification.

MOLECULAR CARCINOGENESIS 32:111–117 (2001)

ARTICLES

Calcium Hydroxyapatite Promotes Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines

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Radiographic mammary microcalcifications are one of the most pertinent diagnostic markers of breast cancer. Breast tissue calcification in the form of calcium hydroxyapatite (HA) is strongly associated with malignant disease. We tested the hypothesis that calcium HA may exert biological effects on surrounding cells, thereby facilitating breast cancer progression. Our findings showed that HA crystals enhanced mitogenesis in breast cancer cell lines MCF-7 and Hs578T and also in normal human mammary epithelial cells. HA crystals were also found to upregulate the production of a variety of matrix metalloproteinases (MMPs), including MMP-2, -9, and -13 in MCF-7 and MMP-9 in human mammary epithelial cell lines. HA crystals were found to greatly augment prostaglandin E_2 levels in Hs578T cells, and treatment with a cyclooxygenase inhibitor, aspirin, abrogated the HA-induced mitogenesis. These results suggest that calcium HA crystals may play an active role in amplifying the pathological process involved in breast cancer.

Key words: microcalcification; prostaglandin; tumorigenesis; calcium phosphate

INTRODUCTION

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Analysis of these microcalcifications by electron microscopy, microprobe analysis, and X-ray diffraction has shown that in breast tissue at least two principle types of calcifications can be distinguished according to their structure and chemical composition [1]. Type I microcalcifications are composed of calcium oxalate in the form of weddellite crystals and type II microcalcifications consist of calcium phosphates in the crystalline form of hydroxyapatite (HA), $Ca_{10}(PO_4)_5(OH)_2$, which is also the basic calcium phosphate found in mature bones and teeth. There is evidence that calcium phosphate and oxalate tend to be associated with different kinds of breast lesions [2]. The presence of oxalate-type microcalcifications appears to be a reliable criterion in favor of the benign nature of the lesion or, at most, of an in situ lobular carcinoma and is rarely associated with malignancy [3,4]. In contrast, the calcifications associated with malignant breast lesions are generally found to be hydroxyapatite [5].

Although their diagnostic value is of great importance radiographically, the genesis of breast calcifications is unclear. The mineralization of breast tissue occurs by deposition of carbonated HA crystals in an extracellular matrix consisting of type I collagen and a variety of noncollagenous proteins. Among these, expression of the bone matrix proteins (bone sialoprotein, osteonectin, osteopontin) and also parathyroid hormone-related protein are believed to play an important role in the initiation and regulation of the deposition of microcalcifications [6]. The luminal calcifications associated with breast lesions appear to be the consequence of an active secretory process by the tumor cells and not solely the result of mineralization of cellular debris and degenerate tumor cells [7]. The occurrence of microcalcifications has not been shown to be significantly associated with age or primary tumor size. However, several studies have

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Abbreviations: HA, hydroxyapatite; MMP, matrix metalloproteinase; PGE_2, prostaglandin E_2; HMEC, human mammary epithelial cell; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; PMA, phorbol myristate acetate; IL-1α, interleukin-1α; SDS, sodium dodecyl sulphate; COX, cyclooxygenase.

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shown that survival of patients with mammographic microcalcification is significantly shorter than those without [8,9]. A recent study by Tabar et al. [10] also showed that the relative hazard of death from breast cancer is five times higher for tumors with casting-type calcifications than that for circular lesions with no calcifications.

The potent biological effects of calcium HA crystals are well recognized in other diseases unrelated to the breast. For example, crystals of basic calcium phosphate (a term used to describe a mixture of predominantly HA, with small amounts of octacalcium phosphate and tricalcium phosphate) are common in osteoarthritic knee effusions. These crystals clearly potentiate joint damage, as their presence and concentration correlates strongly with radiographic evidence and degree of cartilage degeneration [11]. The biological effects of HA crystals which promote articular damage have been well described and include the induction of synoviocyte mitogenesis, accompanied by upregulation of several members of the matrix metalloproteinase (MMP) family leading to marked synovial proliferation and severe cartilage degeneration [12]. These properties may also be relevant in breast oncology.

There have been numerous histological ultrastructure studies of HA deposits in breast carcinomas. However, despite their potent biological effects in other systems and their association with poorer survival in breast cancer patients, to date there have been no investigations of their potential role in the growth and progression of breast tumors. In the present study, we investigated the pathogenic potential of calcium HA crystals in human breast cell lines by studying their ability to induce mitogenesis and upregulate prostaglandin E_2 (PGE_2) and MMP production in MCF-7, Hs578T, and normal human mammary epithelial cells (HMECs).

MATERIALS AND METHODS

Crystal Synthesis and Preparation

HA crystals were synthesized by alkaline hydrolysis of brushite as previously described [13]. Mineral prepared by this method has a calcium:phosphate molar ratio of 1.59 and contains predominately calcium hydroxyapatite as shown by Fourier transform infrared spectroscopy. The crystals were sterilized and rendered pyrogen free by heating at 200°C for 90 min. Crystals were weighed and resuspended by brief sonication in Dulbecco's modified Eagle's medium (DMEM).

Cell Culture

MCF-7 and Hs578T (American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100 mM sodium pyruvate and bovine insulin (4 mg/mL). HMECs and mammary epithelial cell growth media were purchased from Clonetics (Biowhittaker, UK). All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide/95% air.

Stimulation of Cells in Culture

Cells were seeded in 24-well plates at 6×10^4 cells/well and rendered quiescent by incubation in medium containing 0.5% FBS for 24 h. Fresh 0.5% FBS–containing medium was then added and the cells treated with HA crystals (18 μg/cm^2), epidermal growth factor (EGF) (0.1 ng/mL), FBS (10%), phorbol myristate acetate (PMA) (1 μM), interleukin-1α (IL-1α) (2.5 ng/mL), and latex beads (Sigma, St. Louis, MO) (18 μg/cm^2) or left untreated for 8 h (for PGE_2 assay) or 48 h (for cell counts and collection of conditioned medium and cell lysates).

[^{3}H]Thymidine Incorporation Assays

Cells were seeded in 24-well plates at 6×10^4 cells/well and rendered quiescent by incubation in 0.5% FBS for 24 h. [^{3}H]Thymidine (1 μCi/mL) was added to the wells 23 h after the addition of HA crystals (18 μg/cm^2), EGF (0.1 ng/mL), and FBS (10%), latex beads (18 μg/cm^2) or left untreated and pulse-labeled for 1 h. Each condition was performed in quadruplicate. To examine the effect of HA-induced PGE_2 production on mitogenesis, 200 mM aspirin was added to cells 3 h prior to pulse-labeling. The cells were then washed and macromolecules were precipitated with 5% trichloroacetic acid solution. Levels of trichloroacetic acid-precipitable ^{3}H were determined in quadruplicate, using a liquid scintillation counter (Wallac 1214 Rackbeta, Turku, Finland).

Gelatin Zymography

Sodium dodecyl sulphate (SDS)–polyacrylamide gels were prepared with gelatin (1 mg/mL) copolymerized in the 10% resolving gel, and samples containing equal protein concentrations were separated under nondenaturing conditions. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min to remove the SDS and allow the MMPs to renature and then incubated for 24 h at 37°C in 50 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 30 mM $CaCl_2$. Gels were stained with Coomassie R-250 and destained with water.

Western Blots

Samples containing equal protein concentrations were electrophoresed on 10% SDS–polyacrylamide gels, and proteins were electrophoretically transferred to nitrocellulose membranes for 2 h. Membranes were blocked in 2.5% nonfat dry milk. The membranes were then incubated for 3 h with a 1:500 dilution of primary anti–MMP-13 antibody (R4356, a gift from Peter Mitchell, Pfizer-Central

Research Division, Groton, CT). Secondary peroxidase-conjugated anti–rabbit immunoglobulin was used at a dilution of 1:5 000. Immunoreactive bands were detected using enhanced chemiluminescence reagents ECL-plus (Amersham Pharmacia Biotech, UK).

PGE_2 Immunoassay

Cells were incubated in Hanks Hepes buffer with 50 μM arachidonic acid for 15 min and samples collected in duplicate. PGE_2 synthesis was measured using a commercially available PGE_2 immunoassay from R&D Systems (UK). PGE_2 assays were carried out in duplicate three times.

Statistics

Statistical analysis was performed using the Wilcoxon rank sum test.

RESULTS

Calcium HA Enhanced Mitogenesis

We examined the mitogenic effect of treating both malignant and normal mammary cells with HA crystals in vitro. We looked at the effect of the known mitogens, EGF and FBS, on the cells. The mitogenic effect of latex beads, particles of a similar size and concentration to the crystals, was also investigated. Mitogenesis was assessed by $[^3H]$thymidine incorporation assays. In all cell lines the addition of 18 μg/cm^2 HA crystals for 24 h enhanced mitogenesis above untreated control cells. This increase was statistically significant for all cell lines ($P < 0.05$) (Figure 1a). The increase over control unstimulated cultures for HA-treated cells was 1.29-fold for HMECs, 2.08-fold for MCF-7, and 2.28-fold for Hs578T. The mitogenic effect of EGF another known stimulus of epithelial cells was examined (2.25-fold increase for HMECs; 1.35-fold for MCF-7; and 1.94-fold for Hs578T). The cell lines were routinely grown in 10% FBS, which also had a mitogenic effect (4-fold for HMECs; 2.26-fold for MCF-7; and 3.8-fold for Hs578T). Treatment with 18 μg/cm^2 latex beads had no mitogenic effect on the cell lines.

We also performed cell counts using a hemocytometer to confirm that the increased DNA synthesis was accompanied by an increase in cell number. The mitogenic effect of HA crystals shown by increased thymidine incorporation was confirmed by a statistically significant increase in cell numbers 48 h following stimulation ($P < 0.05$) for all cell lines (Figure 1b). The increase in cell numbers over control of cells treated with HA was HMECs, 1.86-fold; MCF-7, 1.93-fold; and Hs578T, 1.94-fold. The increase over control of cells treated with EGF was HMECs, 2.818-fold; MCF-7, 3.62-fold; and Hs578T, 2.16-fold. Treatment of the cells with latex beads had no significant effect on cell numbers.

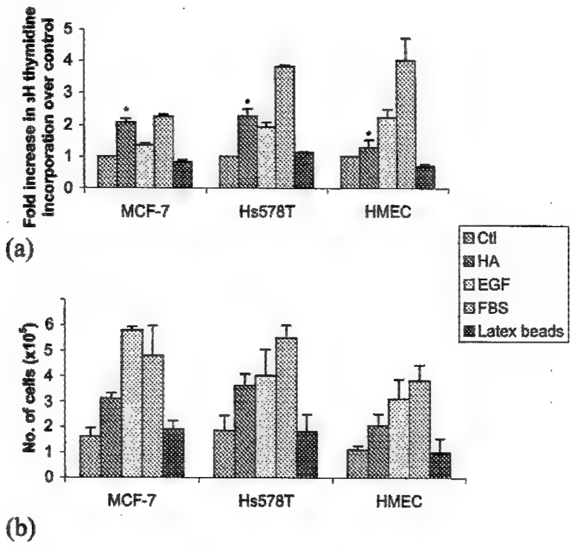

Figure 1. Mitogenic effect of HA crystals on breast cancer cell lines. Confluent, quiescent cultures of MCF-7 and Hs578T cell and HMECs were stimulated with HA crystals (18 μg/cm^2), EGF (0.1 ng/ml), FBS (10%), latex beads (18 μg/cm^2), or left untreated (Ctl). (a) After 23 h cells were pulse-labeled with ^{3}H-thymidine (1 μCi/ml) for 1 h. Levels of trichloroacetic acid–precipitable ^{3}H were determined in quadruplicate, using a liquid scintillation counter. HA caused a statistically significant increase in $[^3H]$ thymidine uptake over untreated cells ($P < 0.05$). All values are given as the mean fold increase over control unstimulated cells ± SEM, n = 4. (b) Cell counts were performed using a hemocytometer following 48-h stimulation. HA also caused a statistically significant increase in cell numbers ($P < 0.05$), n = 4. (*$P < 0.05$).

The mitogenic effect of the HA crystals on the cell lines was further characterized by a dose-response curve. Concentrations of HA ranging from 0 to 42 μg/cm^2 were added to the three cell lines. An increase in the mitogenesis of all three cell lines was seen when treated with increasing concentrations of HA (Figure 2).

Calcium HA Upregulated MMP Production

In this study we investigated the effect of HA crystals on MMP-2 and MMP-9 expression using gelatin zymography and MMP-13 expression in MCF-7 cells by western blotting. Figure 3a shows a

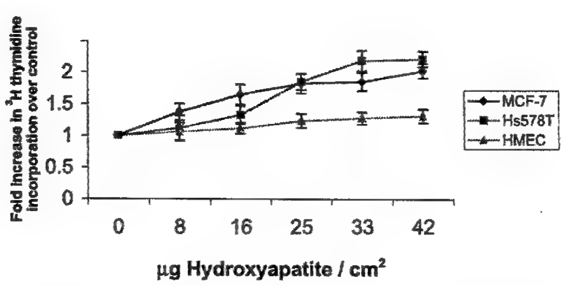

Figure 2. Effect of varying concentrations of HA on $[^3H]$ thymidine incorporation in breast cancer cell lines. Concentrations of HA varying from 0 to 42 μg/cm^2 were added to cell lines MCF-7, Hs578T, and HMEC. All values are given as the mean fold increase over control unstimulated cells ± SEM, n = 4.

zymogram of MCF-7 conditioned medium with bands of lytic activity at 92 and 72 kDa representing MMP-9 and -2, respectively. HA crystals (18 $\mu g/cm^2$) caused upregulation of MMP-2 and MMP-9 activity in MCF-7 cells following 48-h stimulation. HA crystals increased MMP-9 and -2 production over control, untreated levels. EGF and FBS also caused induction of MMP expression in these cells. Gelatin zymography also showed upregulation of MMP-9 activity in HMEC in response to HA crystals (Figure 3b). EGF and FBS also caused an increase in MMP activity in these cells. In contrast, HA stimulation had no effect on MMP-2 or -9 expression in Hs578T cells (data not shown). However, the basal levels of MMP-2 in Hs578T cells were elevated relative to the less invasive MCF-7 cells and Hs578T produced undetectable amounts of MMP-9 by zymography, even after stimulation with EGF or FBS. The lack of induction by HA in the Hs578T cells may also reflect a change of phenotype associated with the more invasive cell type. MMP-13 protein production was also found to be upregulated in MCF-7s when treated with HA crystals (Figure 3c). MMP-13 expression was not examined in Hs578T.

Effect of Calcium HA on PGE_2 Synthesis

We examined the effect of HA crystals on PGE_2 production in breast cancer cell lines. We found that the biologically aggressive, invasive Hs578T cell line had a higher constitutive level of PGE_2 that was approximately four-fold higher than that observed in the MCF-7 cells (Figure 4a). In addition, HA crystals were found to further augment PGE_2 production in Hs578T cells by almost 8-fold, but caused a modest 1.5-fold increase in MCF-7 cells. Similarly, treatment with PMA and IL-1α increased PGE_2 production in Hs578T cells by approximately 23-fold and 20-fold, respectively, with very little effect on MCF-7 cells. Treatment of the cells with 2 mM aspirin, a general cyclooxygenase (COX) inhibitor, blocked the HA-induced increase in mitogenesis of the cells, bringing mitogenesis back to levels seen in untreated cells (Figure 4b).

DISCUSSION

The biological effects of calcium HA crystals on mammary cells were investigated in vitro in our laboratory, and properties of calcium HA were observed that emphasize its pathogenic potential. The first is its ability to promote mitogenesis, possibly amplifying the malignant process by leading to aggravation of tumor growth. In this study we report that HA increased mitogenesis in both normal and malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis, as reported with foreskin fibroblasts [14]. We also showed that HA crystals stimulated mitogenesis of quiescent mammary cell lines in a concentration-dependent fashion. Previously, we have shown that HA crystals stimulate mitogenesis of quiescent cultured human foreskin fibroblasts and adult articular chondrocytes in a concentration-dependent fashion [15]. The mechanism of HA crystal-induced activation of human foreskin fibroblasts involves two processes: a fast membrane-associated event involving protein kinase C and mitogen-activated protein kinase activation, nuclear factor-κB induction, and expression of proto-oncogenes c-*fos* and c-*myc*; and the relatively slow endocytosis and intracellular dissolution of the HA crystals, raising intracellular calcium and causing the activation of a number of calcium-dependent processes leading to cell proliferation [15]. The precise molecular mechanism of HA induced-activation of mammary cells is currently being investigated.

The early proliferative stages of breast cancer are characterized by a continuous basement membrane separating the hyperplastic epithelial cells from the surrounding stroma. Pathologically, the transition from in situ to invasive carcinoma is usually accompanied by interruption of the basement membrane caused by an enhanced process of proteolysis contributing to the escape of breast cancer cells into neighboring tissues, eventually leading to the formation of distant metastases. MMPs are members of a unique family of proteolytic enzymes that can degrade native collagens and other extracellular matrix components [16]. Previous experimental and clinopathological studies have shown good correlations between expression of MMPs and the invasive phenotype of tumor cells [17]. The inducibility of the MMPs by a diverse range of extracellular stimuli has been well documented, including growth factors, phorbal esters, hormones, steroids, and adhesion molecules [16]. Other studies have demonstrated how inappropriate expression of MMPs can initiate a cascade of events that may represent a coordinated program leading to a phenotypic transformation in mammary epithelial cells [18]. The ability of HA crystals to induce members of the MMP family may reflect a similar cascade of events in our model. The differing effects of HA on MMP expression in the cell lines examined in this study may reflect their contrasting states of differentiation. MCF-7 have an epithelial-like phenotype, are estrogen receptor positive, and are weakly invasive, while Hs578T have a more fibroblast-like phenotype, are estrogen receptor negative, and are highly invasive. Studies have shown that HA crystals are potent inducers of MMP-1, -3 and -9 in human foreskin fibroblasts and synoviocytes. Cheung and co-workers [19] have recently shown that HA crystals induce MMP-1 expression through an extracellular-regulated protein kinase 1 and 2 pathway also involving c-fos/AP-1 and Ras signaling pathways.

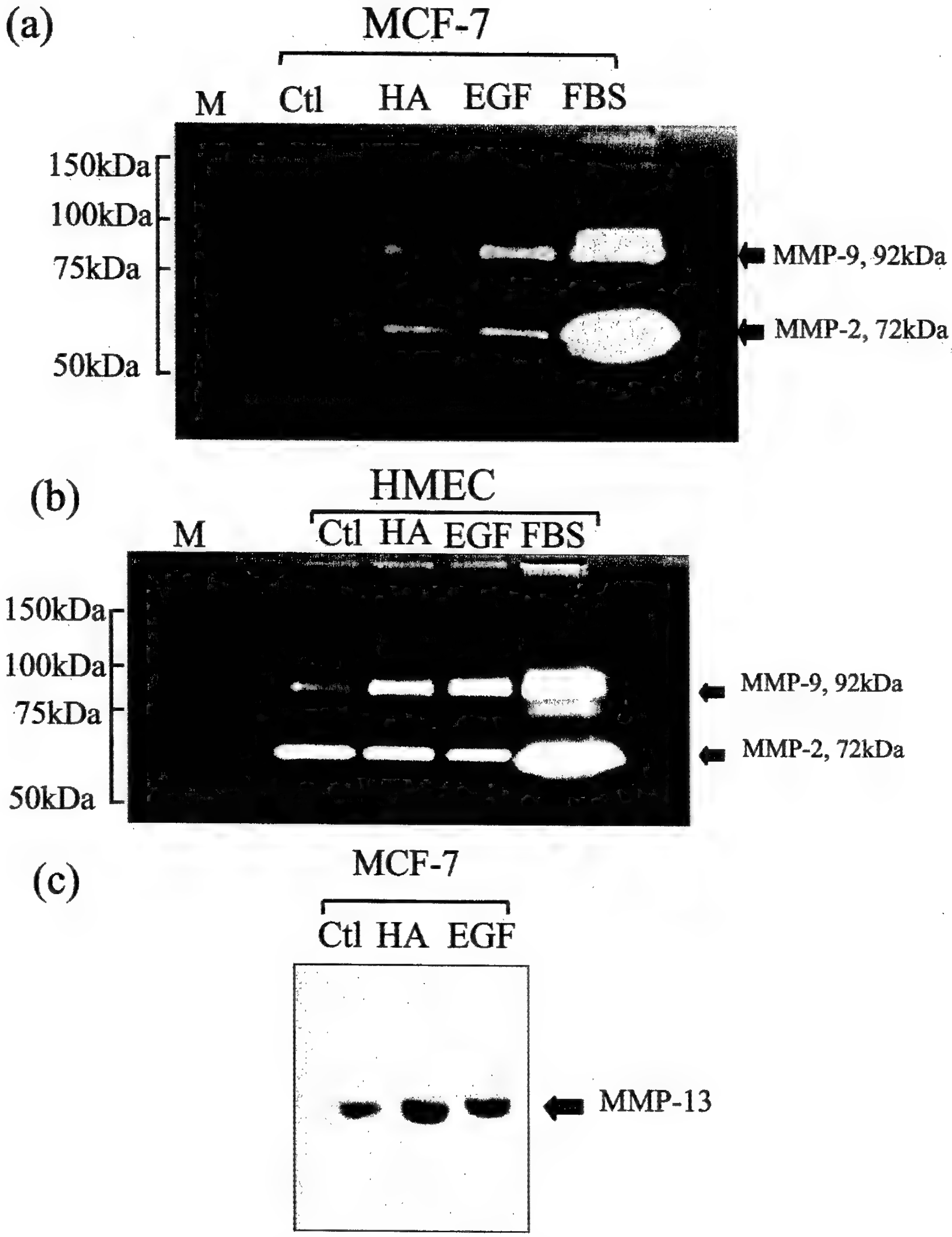

Figure 3. HA crystals induce gelatinase activity in human breast cancer cell lines. (a) MCF-7, (b) HMEC. Confluent, quiescent cells were stimulated with HA crystals (18 $\mu g/cm^2$), EGF (0.1 ng/mL), FBS (10%) or left untreated (Ctl) for 48 h. Conditioned medium was then collected and analyzed by electrophoresis on a 10% polyacrylamide gel containing 1 mg/mL gelatin. After overnight incubation at 37°C, the gels were stained with Coomassie blue. Digestion of the gelatin substrate is seen as a clear band. (c) Effect of HA crystals on MMP-13 protein expression in MCF-7 cells. CM was analyzed by western blotting using a polyclonal antibody to MMP-13 (R4356). The gels and blot shown are representative of duplicate experiments.

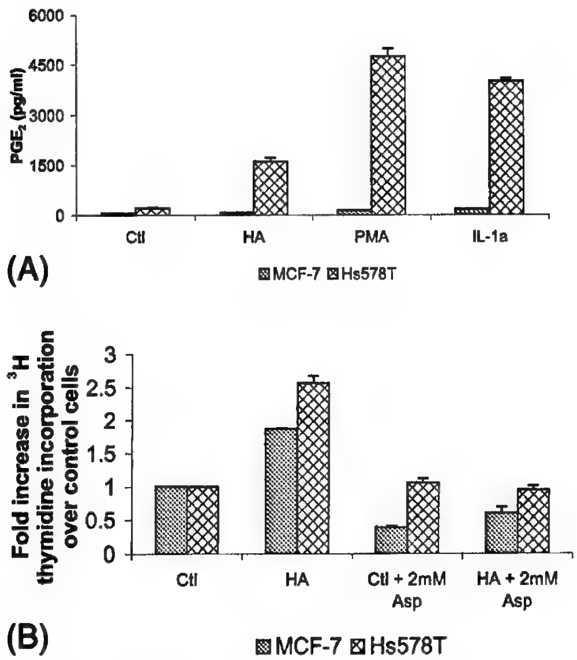

Figure 4. (a) Effect of HA crystals on PGE_2 synthesis in MCF-7 and Hs578T cells. Confluent, quiescent cells were stimulated with HA crystals (18 $\mu g/cm^2$), PMA (1 μM), or IL-1α (2.5 ng/mL) or left untreated (Ctl) for 8 h. All values are given as the mean $\pm$ SEM, $n = 3$. (b) Inhibition of the mitogenic effect of HA crystals on breast cancer cell lines by aspirin. Confluent, quiescent cultures were stimulated with HA crystals (16 $\mu g/cm^2$) or left untreated (Ctl). Aspirin (2 mM) was added to the cells 3 h prior to pulse-labeling with [^{3}H] thymidine. All values are given as the mean fold increase over control unstimulated cells $\pm$ SEM, $n = 4$.

Elevated levels of PGE_2 have been widely reported in many human breast cancers as well as experimental murine mammary tumor models [20]. Several studies with murine mammary tumor cells indicated that PGE_2 may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer [21]. Furthermore, high levels of PGE_2 are often associated with estrogen receptor-negative tumors that exhibit a high metastatic potential [21]. The COX enzymes catalyze the conversion of arachidonic acid to prostaglandins. Calcium-containing crystals have previously been reported to stimulate prostaglandin release from cultured mammalian cells accompanied with the release of proteases. Here we have shown that HA can induce PGE_2 production in tumor cell lines and that treatment with a COX inhibitor, aspirin, can inhibit the HA-induced mitogenic response. In the present study the contrasting expression of PGE_2 in the cell lines MCF-7 and Hs578T may be caused by differential upstream regulation of COX expression. Differential expression and regulation of COX-1 and -2 has been reported in two human breast cancer cell lines (MDA-MB-231 and MCF-7) in which COX-2 expression and induction was reported to be influenced by hormone status and metastatic phenotype [22]. Furthermore, we have also recently shown that HA crystals cause induction of COX-2 mRNA and protein in human fibroblasts [23]. Our results suggest that exposure to HA crystals can cause a significant increase in PGE_2, possibly by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells.

The study presented supports the development of a potentially useful in vitro model system to investigate the HA-dependent modulation of mammary epithelial cells and to our knowledge is the first of its kind reported in the literature. The in vitro exposure used here is to bathe cells with HA crystals, while in vivo exposure appears to involve cell contact with mineralized deposits [24]. However, consistent with our model is the observation that ultrastructural studies demonstrated the presence of HA crystals in breast cancer cells both in their cytoplasm and associated to their membrane [7].

For some time, microcalcifications associated with breast lesions were considered to represent an epiphenomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction and increased PGE_2 synthesis may be part of a program of gene expression designed for malignant growth. These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and emphasize the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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Mechanism of Calcium Hydroxyapatite induced Mitogenesis and Gene Expression in Human Breast Cancer Cell Lines[1]

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Running Title: Calcium Hydroxyapatite Crystals in Breast Cancer.

Keywords: microcalcification, calcium phosphate, matrix metalloproteinase, tumorigenesis.

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The abbreviations used are: HA, hydroxyapatite; MMP, matrix metalloproteinase; PGE_2, prostaglandin E_2; HMEC, human mammary epithelial cells; HFF, human foreskin fibroblasts; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; EGF, epidermal growth factor; PMA, phorbol myristate acetate; IL-1β interleukin-1β; COX, cyclooxygenase; PC, phosphocitrate, ANOVA, analysis of variance, SE, standard error.

ABSTRACT

Microcalcifications are often associated with both benign and malignant human breast lesions. Around 40% of mammary carcinoma present such ectopic mineralization and frequently, they are the only mammographic features that indicate the presence of a tumoural lesion. We previously reported an induction of mitogenesis by HA crystals in normal human mammary epithelial cells and breast cancer cell lines. In the present study we attempted to elucidate the mechanism of this induction. Firstly, we found that direct cell-crystal contact was required for induction of mitogenesis as the effect was not merely a result of isotopic exchange of calcium into the culture medium. Treatment with bafilomycin A_1, a proton pump inhibitor, abrogated HA-induced mitogenesis to control cell levels. These results suggest that phagocytosis and intracellular crystal dissolution is required for HA-induced mitogenesis. The resultant increase in cytoplasmic calcium concentration presumably activates calcium-dependent pathways, which result in mitogenesis. We were able to demonstrate that the increase in PGE_2 and MMP activity previously reported is due to HA mediated upregulation of cyclooxygenase-2 and matrix metalloproteinase-1 at the transcriptional level. An accumulation of interleukin-1β mRNA was also found at 2 and 4hours in response to HA stimulation. Furthermore, we found that phosphocitrate, a naturally occurring inhibitor of HA crystallisation blocked the increase in mitogenesis. These results show that calcium HA crystals are capable of exerting significant biological effects on surrounding cells and could play an active role in amplifying the pathological process involved in breast cancer.

INTRODUCTION

Radiographic mammary microcalcifications occur in 30-50% of breast cancers and constitute one of the most important diagnostic markers of both benign and malignant lesions of the breast. Analysis of microcalcifications by electron microscopy, microprobe analysis and x-ray diffraction has revealed two distinct forms of microcalcifications in breast disease on the basis of their appearance and chemical composition (Harris, Morrow et al. 1993). Oxalate (CaC_2CO_4-$2H_2O$) calcifications (Type I) are generally associated with proliferating but non-invasive diseases of the breast whereas calcium phosphate in the crystalline form of hydroxyapatite (HA) [Ca_{10} (PO_4) 6(OH_2)] is usually correlated to invasive malignant tumors (Radi 1989; Going, Anderson et al. 1990; Frouge, Meunier et al. 1993). Data in the literature suggests that deposition of the bone specific mineral HA results from an active biological process, the mechanism of which has not yet been elucidated. Much attention has been paid to the composition of microcalcifications in breast cancer, but very little attention has been paid to the possibility that the deposition of calcium in breast cancers might represent a biologically significant feature of selected tumours. Mammographic microcalcification does not appear to be significantly associated with age or primary tumour size. Lymph node involvement by tumour is present in 50% of patients with mammographic microcalcification in relation to the primary tumour, but only 24% of patients without microcalcification (Holme, Reis et al. 1993). Tabar *et al.* reported that the only reliable discriminating criterion in small mammographically detected tumors of 1-14mm is the presence of casting type calcifications on mammography and not the traditional prognostic features of node status or malignancy grade (Tabar, Chen et al. 2000). Tabar *et al.* also demonstrated that the relative hazard of death from breast cancer for women with small mammographically detected breast cancers was five times higher for tumors with casting-type calcifications than that for circular lesions without calcification.

In an earlier *in vitro* study, we reported properties of calcium HA in human mammary cell lines which emphasise the pathogenic potential of these microcalcifications and their relevance in breast oncology (Morgan, Cooke et al. 2001). HA crystals were shown to induce mitogenesis of quiescent normal and malignant mammary cell lines in a dose-dependent manner and to upregulate gelatinase activity and prostaglandin E_2 (PGE_2) production. In the present study we attempt to elucidate the molecular mechanism of HA-induced activation of mammary cell lines. Using an *in vitro* model system with Hs578T and MCF-7 adenocarcinoma cell lines we investigated whether direct cell-crystal contact, followed by phagocytoses and intracellular dissolution is required for crystal-induced mitogenesis. We examined the possibility of attenuating the mitogenic response to HA by treating the cells with bafilomycin A_1 (a specific inhibitor of the vacuolar type proton pump ATPase) and phosphocitrate (PC), a naturally occurring inhibitor of HA crystallisation. Following on from our previous study we examined whether the increase in PGE_2 and gelatinase activity was due to HA mediated upregulation of cyclooxygenase-2 (COX-2) and matrix metalloproteinase-1 (MMP-1) at the transcriptional level. The accumulation of interleukin-1β (IL-1β) mRNA in response to HA stimulation was also studied.

MATERIALS AND METHODS

Hydroxyapatite Crystal Preparation

HA crystals were synthesised by alkaline hydrolysis of brushite, using a modification of the method of Bett *et al.*,(Bett, Christener et al. 1967). The ^{45}Ca-HA crystals were prepared as described previously using calcium-45 (Amersham Pharmacia Biotech, UK) (McCarthy, Cheung et al. 1998). Mineral prepared by this method has a calcium / phosphate molar ratio of 1.59 and contains partially carbonate–substituted hydroxyapatite mixed with octacalcium phosphate as determined by Fourier transform infrared spectroscopy. The crystals were weighed into vials and rendered pyrogen-free by heating at 200°C for 90min. The sterile crystals were resuspended by brief sonication in Dulbecco's modified Eagle's medium (DMEM).

Cell Culture

MCF-7 and Hs578T (American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin, 1mM sodium pyruvate and 10μg/ml bovine insulin. Human foreskin fibroblast (HF) cells and fibroblast basal medium were purchased from Clonetics (Biowhittaker, UK). All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide / 95% air.

[^{3}H] Thymidine Incorporation Assays

Cells were seeded in 24-well plates (Corning, Costar) at 6 x 10^4 cells / well and rendered quiescent by incubating in 0.5% FBS for 24 hr. Fresh serum free media with or without bafilomycin A_1 (2nM and 5nM) or phosphocitrate (1mM) was added to HF Hs578T or MCF-7 cells. Cells were incubated for 30min and subsequently stimulated with HA (18μg/cm^2) for 23hr, HA-free controls were also prepared. Phosphocitrate was a kind gift from John D. Sallis, University of Tasmania, Australia. Each condition was performed in quadruplicate. Following incubation [^{3}H] thymidine (1μ Ci/ml) was added to each well and pulse labelled for 1hr. The cells were then washed and macromolecules precipitated with 5% trichloroacetic acid solution. Levels of trichloroacetic acid-precipitable [^{3}H] thymidine were determined using a liquid scintillation counter (Wallac 1214 Rackbeta, Turku, Finland).

To determine if direct cell-crystal contact is required for HA-induced mitogenesis, confluent, quiescent cultures of MCF-7 (a) and Hs578T (b) cells were stimulated with HA crystals (18μg/cm^2), left unstimulated or crystals were added above the cells to sterile membrane inserts with 8μm pore size (Falcon), preventing cell-crystal contact but allowing exchange of any dissolved ions. After 23h cells were pulse-labeled with [^{3}H] thymidine (1μCi/ml) for 1h. Levels of trichloroacetic acid-precipitable [^{3}H] thymidine were determined in quadruplicate using a liquid scintillation counter as above.

Determination of Intracellular Dissolution of ^{45}Ca-Labelled HA Crystals

Intracellular dissolution of ^{45}Ca labelled HA crystals was determined as described previously (McCarthy, Cheung et al. 1998). Briefly, confluent, quiescent Hs578T and MCF-7 cultures were treated with varying concentrations of bafilomycin A_1, and

incubated with ^{45}Ca-labelled HA ($18\mu g/cm^2$) for 48hr. Control cultures were incubated with ^{45}Ca-labelled HA crystals alone. In addition, to account for non-specific ^{45}Ca release, due to isotopic exchange of crystal surface ^{45}Ca with culture media, cell-free wells containing fresh media were prepared and incubated similarly as a further control. After 48hr the media from each well was removed and centrifuged at 5000rpm/5min (Eppendorf Centrifuge 5415R) and the radioactivity in an aliquot of supernatant was measured in a liquid scintillation counter (Wallac 1214, Rackbeta, Turku, Finland). The percent release of ^{45}Ca resulting from intracellular dissolution of ^{45}Ca-HA was calculated in the presence or absence of various concentrations of bafilomycin A_1.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The effect of HA on expression of cyclooxygenase-2 (COX-2), matrix metalloproteinase-1 (MMP-1) and Interleukin-1β (IL-1β) mRNA levels in MCF-7 and Hs578T cells was examined by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Each pair of sense and antisense primers were designed to span at least one intron of the gene to exclude contaminating genomic DNA. Primers used were: GAPDH 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense) and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (antisense); COX-2 5′-TCC TTG CTG TTC CCA CCC ATG-3′ (sense) and 5′-CAT CAT CAG ACC AGG CAC CAG-3′ (antisense); MMP-1 5′-GGT GAT GAA GCA GCC CAG-3′(sense) and 5′-CAG TAG AAT GGG AGA GTC-3′ (antisense). The IL-1β primer pair was purchased from R & D Systems, Inc. (UK). Total RNA isolated using Tri-Reagent (Sigma, UK) was reverse transcribed into cDNA and this was used as the template for the PCR. RNA samples of 1μg were denatured at 70°C for 10min. 200ng of random hexamers, 1 X reverse transcription buffer, 25μM deoxyribonucleoside triphosphates, and 200 units of Moloney murine leukaemia virus reverse transcriptase were added and incubated at 37°C overnight in a reaction volume of 20μl. The reverse transcription reaction was stopped by heating to 95°C for 2min. A 1:10 volume of the generated cDNA reaction was used in the subsequent amplification reaction. For COX-2, MMP-1 and GAPDH, PCR was performed in a 50μl volume with 1.5mM magnesium chloride, 0.1mM deoxyribonucleoside triphosphates, 0.5μM of each sense and antisense primer, and 2.5 units of *Taq* Polymerase. The reaction cycles were denaturing at 94°C for 1 min, annealing at 55°C for GAPDH and COX-2, and 58°C for MMP-1 for 1min, and extension at 72°C for 1 min. The PCR reaction for IL-1β was performed in accordance with manufacturer's instructions. Products were electrophoresed alongside 100Bp DNA ladder (Promega) in 1.2% agarose gels containing ethidium bromide. Expected product size for each of the amplification reactions were MMP-1 (438bp), COX-2 (847bp), GAPDH (550bp), IL-1β (433bp) and IL-1β positive control (320bp).

Statistical Analysis

All quantitative data are expressed as mean ± S.E. The data were analysed by one way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post test. The data for figure 1 was analysed by parametric, unpaired student's *t* test.

RESULTS

Direct crystal-cell contact is required for mitogenic effects of hydroxyapatite (HA) crystals in breast cancer cell lines.

Confluent, quiescent cultures of MCF-7 and Hs578T cells were stimulated with HA crystals ($18\mu g/cm^2$), left unstimulated or crystals were added above the cells to sterile membrane inserts with 8μm pore size, preventing cell-crystal contact but allowing exchange of any dissolved ions. The treatment of MCF-7 and Hs578T cells with hydroxyapatite crystals ($18\mu g/cm^2$) induced a 1.83 and 2.91 fold increase respectively in [^{3}H] thymidine incorporation, an index of mitogenesis, when compared with untreated controls. This induction was statistically significant for both cell lines ($p< 0.001$). Treatment of cells with latex beads ($18\mu g/cm^2$), non-calcific particles of similar size had no mitogenic effect on Hs578T and MCF-7 cells (data not shown). The induction in mitogenesis seen when HA was in contact with the cells was attenuated when crystals were prevented making direct contact with cells by the presence of the porous membrane inserts ($, $p<0.001$ versus HA-treated, figure 1).

Inhibition of Hydroxyapatite Crystal-Induced Mitogenesis by Bafilomycin A_1 in Breast Cancer Cells.

Vacuolar-type H^+-APTase is responsible for acidification in vacuolar compartments such as lysosomes. A specific inhibitor of vacuolar-type H^+-ATPase, bafilomycin A_1, is a macrolide antibiotic which inhibits acidification in lysosomes of cultured cells. Bafilomycin A_1 (2nM and 5nM) significantly ($p<0.001$) inhibited the mitogenic response of Hs578T and MCF-7 to HA crystals ($18\mu g/cm^2$) in a concentration dependent manner while having no significant effect on untreated control cells (figure 2). Thus, raising lysosomal pH to inhibit intracellular HA dissolution attenuates the proliferative response to HA crystals in breast cancer cells.

Effect of Bafilomycin A_1 on ^{45}Ca Release from ^{45}Ca-HA by Hs578T and MCF-7 Cells

We tested the hypothesis that Hs578T and MCF-7 cells solubilise HA crystals and that intracellular dissolution results in the release of free calcium into the culture media (CM). ^{45}Ca labelled HA crystals were synthesised and the cells treated with $18\mu g/cm^2$ ^{45}Ca-HA. We demonstrated an 8% increase of ^{45}Ca release from ^{45}Ca-HA-treated Hs578T cells when compared with isotopic exchange of ^{45}Ca with media in cell-free control wells after 48hr (Figure 3). When cells were treated with bafilomycin A_1 significant inhibition (**, $p<0.01$; ***, $p<0.001$) of ^{45}Ca release into CM was recorded at all concentrations tested (1-10nM). Inhibition of intracellular crystal dissolution by bafilomycin A_1 was dose dependent (figure 3).

Effect of Phosphocitrate on Hydroxyapatite Crystal-Induced Mitogenesis in HF, Hs578T and MCF-7 cells

We examined the effect of phosphocitrate on HA crystal-induced cell proliferation in HF, Hs578T and MCF-7 cell lines by [^{3}H] thymidine incorporation assay. HA crystals ($18\mu g/cm^2$) stimulated a significant increase ($p>0.001$) in mitogenesis in all cell lines when compared to untreated controls (Figure 4). Phosphocitrate (1mM) an inhibitor of hydroxyapatite crystallisation, prevented this induction ($p>0.001$). Inhibition was specific for HA crystal-induced mitogenesis as phosphocitrate had no significant effect on basal (0.5% FBS) or serum-induced (10% FBS) mitogenesis (data not shown).

Effect of Hydroxyapatite on Gene Expression in Breast Cancer Cells

In this study we investigated the effect of HA crystals on COX-2, MMP-1 and IL-1β mRNA expression in Hs578T and MCF-7 cells by RT-PCR analysis. As shown in Figure 5a, treatment with varying concentrations of HA (0.18-180μg/cm^2) for 24hr caused a dose-dependent increase in COX-2 mRNA expression in Hs578T cells. Treatment of Hs578T cells with non-calcific, latex beads failed to promote induction of the COX-2 mRNA transcript. Similarly in figure 5b HA (18μg/cm^2) crystal stimulation is shown to upregulate COX-2 mRNA transcript levels over a range of time-points with optimal expression at 4-8hr, when compared with untreated controls. RNA collected from phorbol 12-myristate 13-acetate (1μM) (known inducer of COX-2 and MMP-1) treated HF cells was used as a positive control (Fig. 5a & Fig 6a). In contrast, the MCF-7 cell line lacked detectable expression of COX-2 transcripts using RT-PCR methodology at any of the time points and HA concentrations tested (data not shown).

Similarly we examined MMP-1 and IL-1β mRNA transcript expression. Hs578T and MCF-7 cells were left untreated or were stimulated with HA crystals (18μg/cm^2). RNA was harvested at various time points from 2-32hr, subjected to RT-PCR analysis and levels of MMP-1 and IL-1β mRNA transcript expression visualised by agarose gel electrophoresis of amplification products. HA crystals induced expression of MMP-1 mRNA within 2.5hr and levels continued to increase at the 4hr and 8hr time-points until a peak was reached at 24hr (Fig. 6a). When Hs578T cells were treated with HA crystals, basal levels of IL-1β mRNA were elevated at the 2hr and 4hr analysis points. However, by 24hr and 31hr, control and HA treated IL-1β mRNA levels were almost equal in intensity. In contrast, HA treatment had no effect on MMP-1 expression in the less invasive MCF-7 cell type, and no endogenous or inducible levels of IL-1β were detected in MCF-7 cells in this study (data not shown).

DISCUSSION

Although the presence of microcalcification has long been used as a radiological indicator only recently has it been recognised that the composition (oxalate vs. phosphate) of breast calcifications may give an idea as to their origin and that certain compositions may be more strongly associated with cancerous conditions (Radi 1989; Frouge, Meunier et al. 1993) and disease outcome (Tabar, Chen et al. 2000). We have shown that HA crystals are capable of exerting significant biological effects on surrounding cells. The study presented here uses a previously described *in vitro* model system to investigate the HA-dependent modulation of mammary epithelial cells, which involves bathing the cells with HA crystals (Morgan, Cooke et al. 2001).

We previously reported an induction of mitogenesis by HA crystals in normal human mammary epithelial cells and breast cancer cell lines. In the present study we sought to elucidate the mechanism of this induction. Firstly, we found that direct cell-crystal contact was required for induction of mitogenesis. When HA crystals were added, but cells were not directly in contact with HA, no induction of mitogenesis was seen. Thus, the effect was not merely the result of isotopic exchange of calcium into the culture medium resulting from the addition of the HA crystals. Using the proton pump inhibitor bafilomycin A_1 to inhibit intracellular dissolution of HA crystals, the data described here also confirm the importance of intracellular dissolution. Treatment with bafilomycin A_1 abrogated HA-induced mitogenesis to control cell levels. These results suggest that phagocytosis and intracellular crystal dissolution is required for HA-induced mitogenesis. The resultant increase in cytoplasmic calcium concentration presumably activates calcium-dependent pathways which result in mitogenesis. Understanding the mechanisms by which HA crystals cause mitogenesis and regulate gene expression might suggest potential effective pharmacological interventions.

Proteolytic enzymes have been repeatedly associated with a metastatic phenotype. The MMPs are associated with degradation of the extracellular matrix (ECM), including the basement membrane, which is composed of collagen, laminin, entactin, and proteoglycans (Nelson, Fingleton et al. 2000). Numerous studies have demonstrated how inappropriate expression of MMPs can initiate a cascade of events that may represent a coordinated program leading to a phenotypic transformation in mammary epithelial cells and demonstrated correlation between expression of MMPs and the invasive phenotype of the tumour cells (Lochter, Galosy et al. 1997). The ability of HA crystals to induce members of the MMP family at the transcriptional level may reflect a similar cascade of events in our model. Studies have shown that basic calcium phosphate crystals are potent inducers of MMP-1, –3 and –9 in human foreskin fibroblasts and synoviocytes. Cheung and co-workers have shown that basic calcium phosphate crystals induce MMP-1 expression in human fibroblasts through an extracellular regulated protein kinase 1 and 2 pathway also involving c-fos/AP-1 and RAS signalling pathways (Brogley, Cruz et al. 1999).

High levels of PGE_2 are often associated with estrogen receptor-negative tumours that exhibit a high metastatic potential (Rolland, Martrin et al. 1980) and several studies with murine mammary tumour cells indicate that PGE_2 may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer (Rolland, Martrin et al. 1980). We previously reported differential PGE_2 production from breast cancer cell lines MCF-7 and Hs578T, which could be increased by HA treatment (Morgan, Cooke et al. 2001). The COX enzymes (COX-1 and –2) catalyze the conversion of arachidonic acid to prostaglandins. In the present

study we have shown that the contrasting production of PGE_2 in the cell lines MCF-7 and Hs578T in response to treatment with HA is caused by differential upstream regulation of COX-2 mRNA expression. Differential expression and regulation of COX–2 has been reported in the literature to be influenced by hormone status and metastatic phenotype in human breast cancer cell lines (Lui and Rose 1996). Our results confirm that exposure to HA crystals can cause a significant increase in PGE_2 by direct induction of COX-2 mRNA.

Although mainly synthesised by monocytes and macrophages Il-1 is known to have a role in tumour invasion including proliferation, cell adhesion and immunomodulation. IL-1 has been found in several human tumours and may enhance tumour invasiveness (Lauri, Bertomeu et al. 1990), perhaps by increasing adhesion to the vascular surface (Lauri, Bertomeu et al. 1990), stimulating tumour cell motility (Verhasselt, Van Damme et al. 1992) and inducing MMP-1 and PGE_2 production. The induction of IL-1β by HA reported here could be functioning in an autocrine loop increasing transcription of MMP-1 and/or COX-2, both of which are well documented to be regulated by IL-1, although further study would be required to prove this. In addition, IL-1 has been shown to increase the stability of several mRNAs in various cell types including MMP-1 mRNA in human fibroblasts and could be functioning in this manner (Rutter, Benbow et al. 1997). The differences in transcriptional activation by HA crystals in Hs578Ts and MCF-7s most likely reflects the complement of transcription factors present within the two cell lines.

Phosphocitrate is a naturally occurring compound which has been identified in mammalian mitochondria and crab hepatopancreas (Tew, Malis et al. 1981). It has been speculated that PC may have an important role in preventing calcium phosphate precipitation in cells or cellular compartments which maintain high concentrations of calcium and phosphate (Tew, Malis et al. 1981). PC prevents soft tissue calcification *in vivo* and does not produce any significant toxic side effects in rats or mice when given in doses up to 150μmol/kg/day (Shankar, Crowden et al. 1984).Other studies in human fibroblasts *in vitro* have shown that PC specifically inhibits calcium pyrophosphate dihydrate and basic calcium phosphate crystal-induced proto-oncogenes (c-*fos* and c-*jun*) expression, mitogen-activated protein kinase activation, metalloproteinase synthesis and mitogenesis, while PC has no effect on similar biological responses induced by epidermal growth factor or serum (Cheung, Salis et al. 1996; Nair, Misra et al. 1997). Cheung *et al.* suggests that the mechanism of action of PC involves the binding of PC to calcium-containing crystals which changes the ζ potential of the crystal surface, thus interfering with the crystal-membrane interactions that lead to cellular responses (Nair, Misra et al. 1997). This would explain why PC does not inhibit peptide growth factor induction of mitogenesis or metalloproteinase synthesis. Phosphocitrate is currently being considered as a potential therapeutic strategy for crystal deposition disease in articular tissues (Cheung 2001) but with its anti-mineralization effect *in vivo* PC could perhaps be considered as a potential therapeutic compound for preventing calcification of the breast.

HA crystals have potent biological activities all of which are consistent with their association with malignant disease. The data presented here further elucidates the mechanism of HA crystal-induction of mitogenesis, PGE_2, MMP-1 and IL-1β.

FIGURE LEGENDS

Figure 1:

Direct crystal-cell contact is required for mitogenic effects of hydroxyapatite (HA) crystals in breast cancer cell lines: [^{3}H] thymidine incorporation. Confluent, quiescent cultures of MCF-7 (A) and Hs578T (B) cells were stimulated with HA crystals (18μg/cm^2), left unstimulated or crystals were added above the cells to sterile membrane inserts with 8μm pore size, preventing cell-crystal contact but allowing exchange of any dissolved ions. After 23h cells were pulse-labeled with [^{3}H] thymidine (1μCi/ml) for 1h. Levels of trichloroacetic acid-precipitable [^{3}H] thymidine were determined in quadruplicate using a liquid scintillation counter. HA caused a statistically significant increase in [^{3}H] uptake (***, $p<0.001$ versus control). This induction was attenuated when crystals were prevented making direct contact with cells ($, $p<0.001$ versus HA-treated). The data are expressed in disintegrations per minute (DPM) as the mean $\pm$ S.E.M.; $n = 4$.

Figure 2:

Inhibition of the mitogenic effects of hydroxyapatite (HA) crystals by bafilomycin A_1 in breast cancer cell lines: [^{3}H] thymidine incorporation. Confluent, quiescent cultures of MCF-7 (A) and Hs578T (B) cells were stimulated with HA crystals (18μg/cm^2) or left unstimulated and treated with bafilomycin A_1 (2nM and 5nM). After 23h cells were pulse-labeled with [^{3}H] thymidine (1μCi/ml) for 1h. Levels of trichloroacetic acid-precipitable [^{3}H] thymidine were determined in quadruplicate using a liquid scintillation counter. HA caused a statistically significant increase in [^{3}H] uptake (***, $p<0.001$ versus control). This induction was attenuated by the specific vacuolar pump inhibitor, bafilomycin A_1 ($, $p<0.001$ versus HA-treated). The data are expressed in disintegrations per minute (DPM) as the mean $\pm$ S.E.M.; $n = 4$.

Figure 3:

Effect of bafilomycin A_1 on ^{45}Ca release from ^{45}Ca–HA by the breast cancer cell lines Hs578T (a) and MCF-7 (b). Confluent, quiescent cultures were incubated with ^{45}Ca-labelled hydroxyapatite crystals (18μg/cm^2) in the presence or absence of varying doses of bafilomycin A_1 for 48h. The amount of free ^{45}Ca released into culture medium was determined using a scintillation counter and expressed as a percentage of total ^{45}Ca added. There was a significant difference in ^{45}Ca release by untreated Hs578T versus Hs578T treated with bafilomycin A_1 (**, $p<0.01$ ***, $p<0.001$) $n = 4$.

Figure 4:

Inhibitory effect of phosphocitrate (1mM) on hydroxyapatite (HA) crystal-induced mitogenesis in HFF (a), Hs578T (b) and MCF-7(c) cell lines. Cells were grown to confluence in 24-multiwell culture plates and rendered quiescent by incubating in 0.5% FBS for 24h. Cells were left untreated or stimulated with HA (18μg/cm^2) in the presence or absence of phosphocitrate (1mM). After 23h all plates were pulse-labeled with [^{3}H] thymidine (1μCi/ml) for 1h. The plates were then processed and trichloroacetic acid-precipitable radioactivity was determined using a liquid scintillation counter. Results are expressed as [^{3}H] thymidine uptake measured in disintegrations per minute. Hydroxyapatite crystals caused a significant increase (***, $p<0.001$ versus control) in cell proliferation in each cell line. Treating the cells

with phosphocitrate (1mM) inhibited the mitogenic response to hydroxyapatite ($, $p<0.001$ versus HA). All values are given as the mean + S.E.M. $n = 4$.

Figure 5:

Hydroxyapatite crystals induce COX-2 mRNA expression in the breast adenocarcinoma cell line Hs578T. Cells were rendered quiescent by incubating in 0.5% FBS for 24h. (a) Hs578T cells were stimulated with various concentrations of hydroxyapatite crystals (HA) (0.18-180μg/cm^2), Latex beads (LB) (18μg/cm^2) or left untreated for 24h.

(b) Cells were stimulated with hydroxyapatite crystals (18μg/cm^2) or left as untreated controls (C) and incubated for various lengths of time.

At the time points indicated mRNA was harvested and transcript levels of COX-2 were determined by RT-PCR analysis followed by agarose gel (1.2%) electrophoresis of amplification products. The housekeeping gene GAPDH was used as an internal control.

Figure 6:

Time course showing induction of MMP-1 and IL-1β by hydroxyapatite crystals in Hs578T cells. Confluent, quiescent Hs578T cells were incubated with hydroxyapatite crystals (HA) (18μg/cm^2) or left untreated (C) over a range of time points (2-30hr). At each time point mRNA transcript levels of MMP-1 and IL-1β were determined by RT-PCR analysis.

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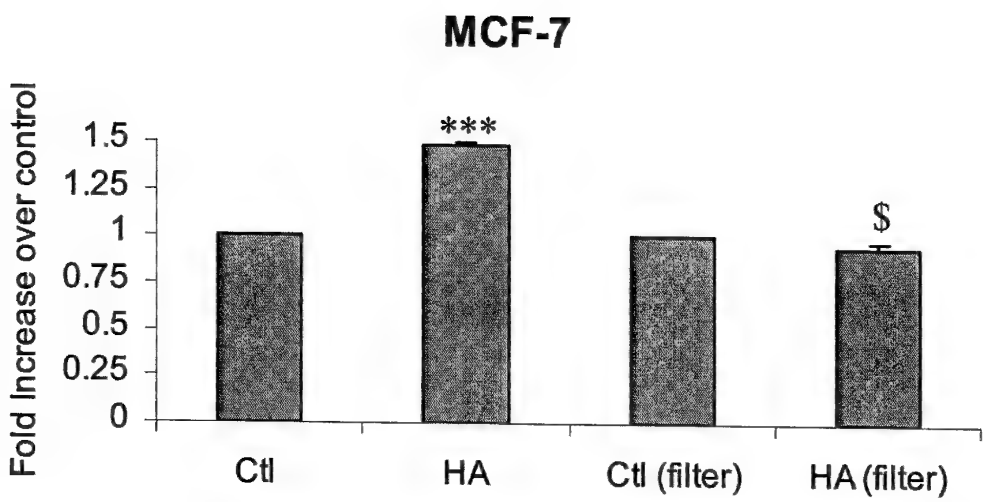
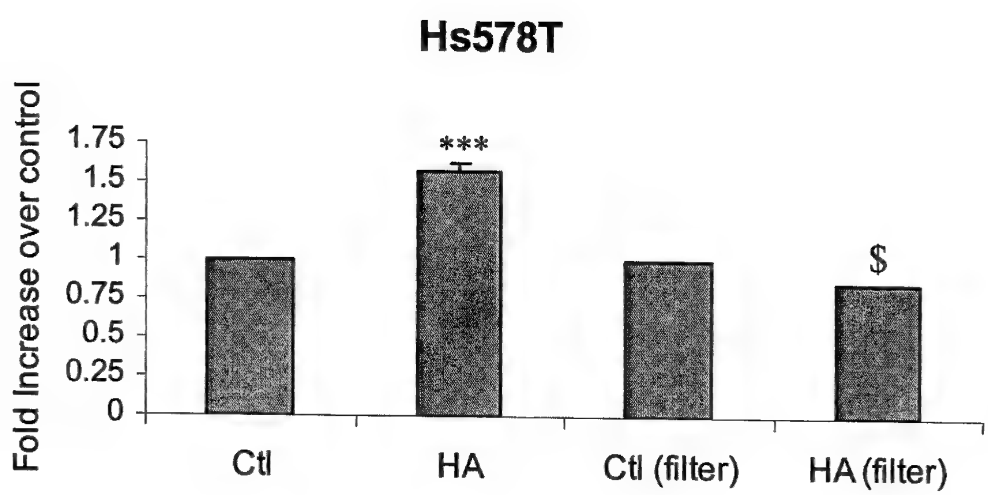
(a)



MCF-7
Fold Increase over control
1.5
1.25
1
0.75
0.5
0.25
0
***
$
Ctl
HA
Ctl (filter)
HA (filter)

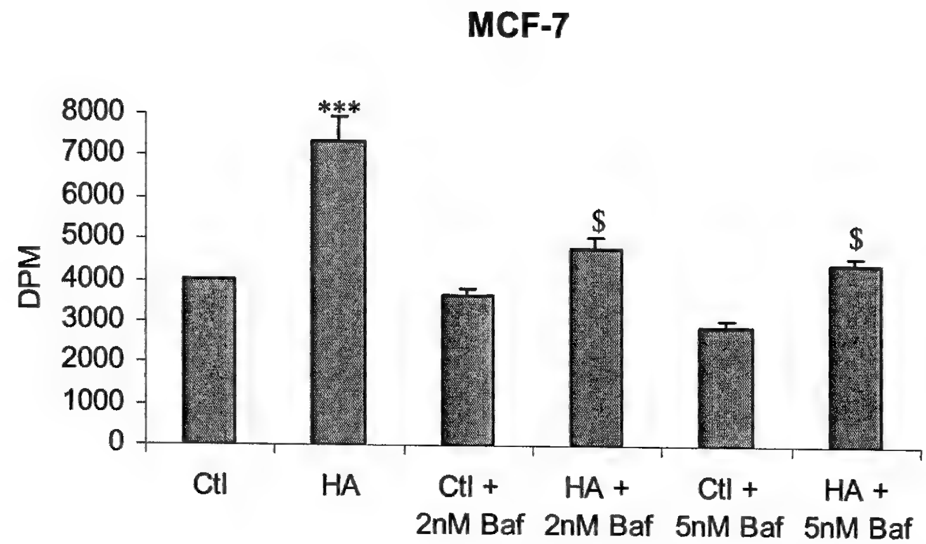
(b)



Hs578T
Fold Increase over control
1.75
1.5
1.25
1
0.75
0.5
0.25
0
***
$
Ctl
HA
Ctl (filter)
HA (filter)

Fig 1

(a)


MCF-7
DPM
8000
7000
6000
5000
4000
3000
2000
1000
0
***
$
$
Ctl
HA
Ctl + 2nM Baf
HA + 2nM Baf
Ctl + 5nM Baf
HA + 5nM Baf

(b)

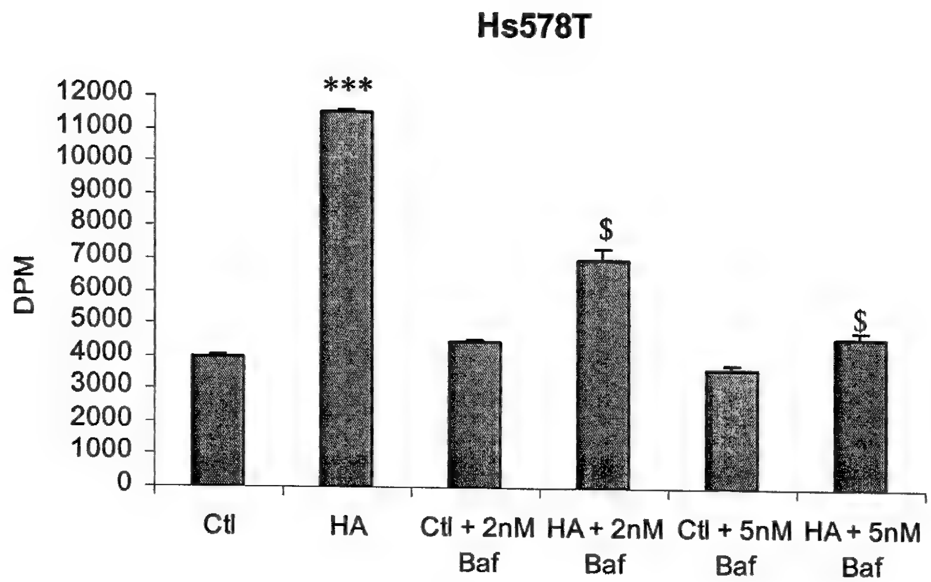
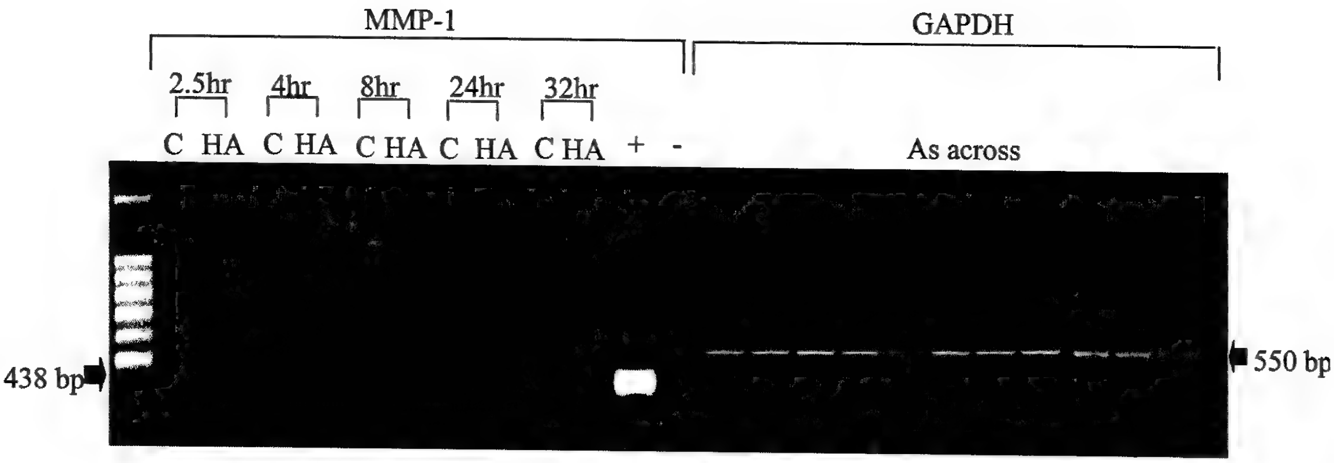

Hs578T
DPM
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
$
$
Ctl
HA
Ctl + 2nM Baf
HA + 2nM Baf
Ctl + 5nM Baf
HA + 5nM Baf

Fig 2

(a)


MMP-1
GAPDH
2.5hr
4hr
8hr
24hr
32hr
C HA C HA C HA C HA C HA + -
As across
438 bp
550 bp

(b)

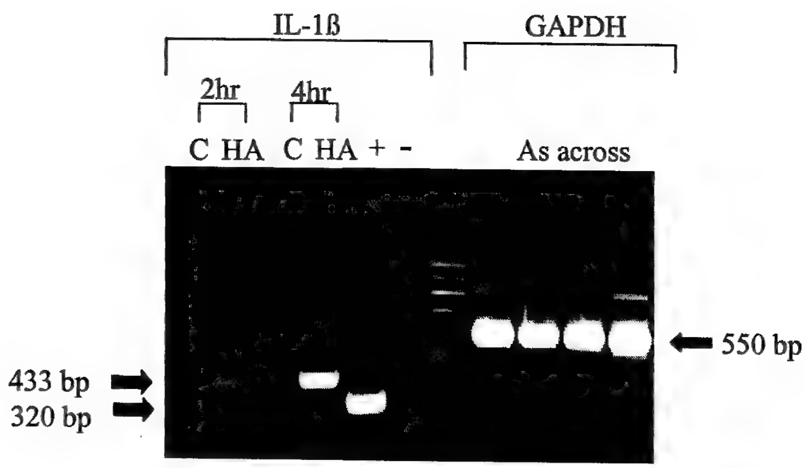
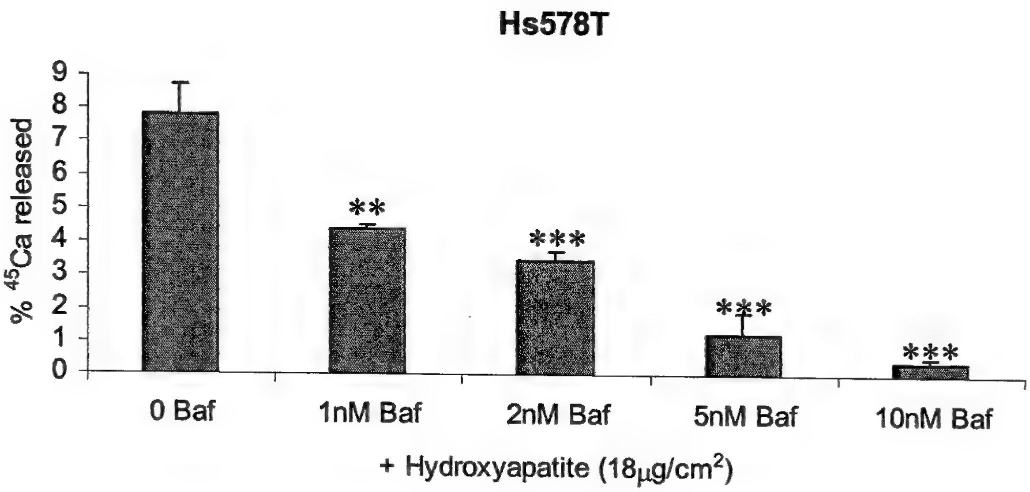

IL-1ß
GAPDH
2hr
4hr
C HA C HA + -
As across
433 bp
320 bp
550 bp

Fig 6

(a)


Hs578T
% ⁴⁵Ca released
9
8
7
6
5
4
3
2
1
0
**
***
***
***
0 Baf
1nM Baf
2nM Baf
5nM Baf
10nM Baf
+ Hydroxyapatite (18μg/cm2)

(b)

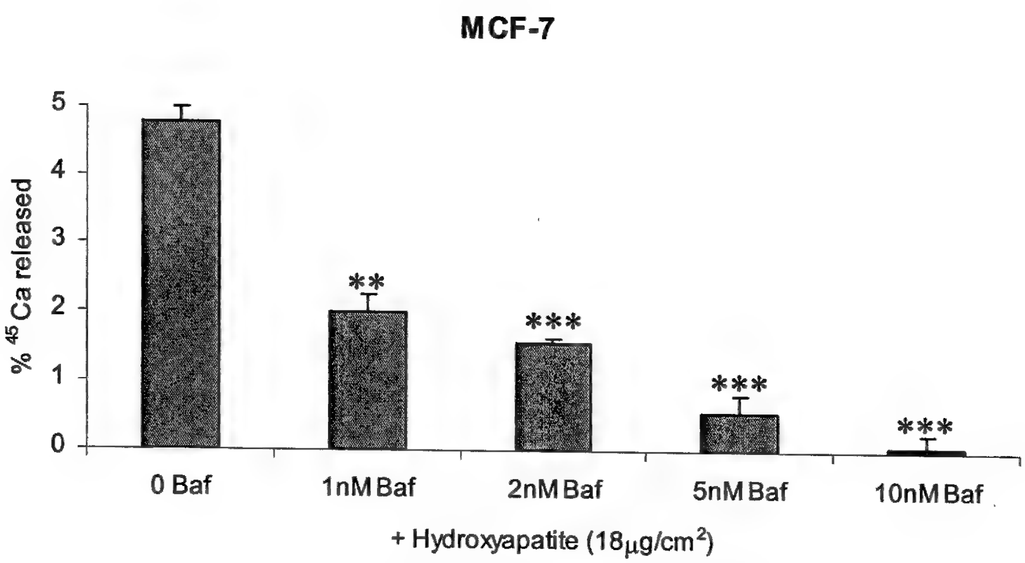

MCF-7
% 45 Ca released
5
4
3
2
1
0
**
***
***
***
0 Baf
1nM Baf
2nM Baf
5nM Baf
10nM Baf
+ Hydroxyapatite (18μg/cm2)

Fig 3

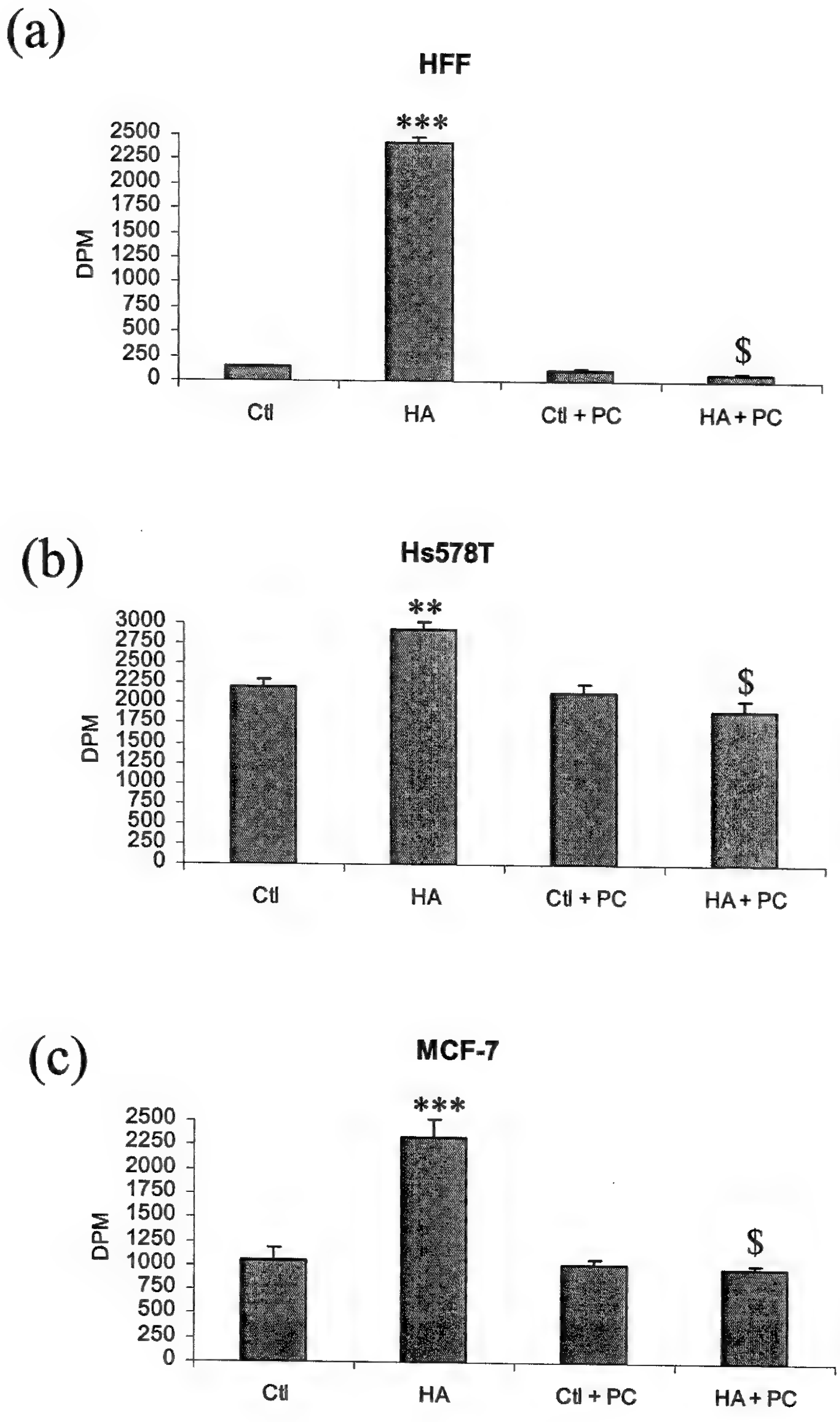
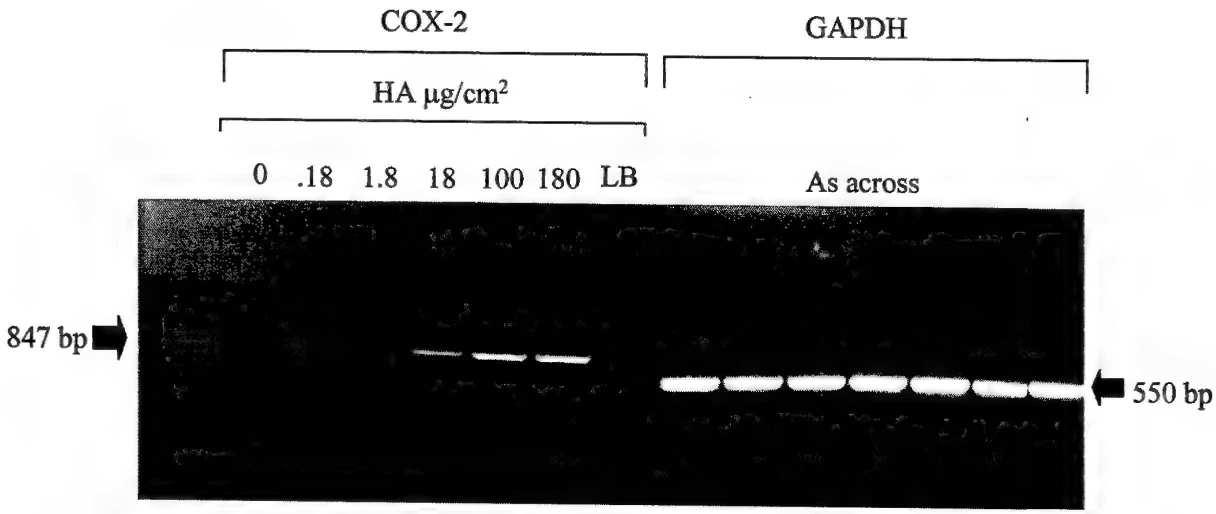

(a)
HFF
***
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
DPM
$
Ctl
HA
Ctl + PC
HA + PC
(b)
Hs578T
**
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
DPM
$
Ctl
HA
Ctl + PC
HA + PC
(c)
MCF-7
***
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
DPM
$
Ctl
HA
Ctl + PC
HA + PC

Fig 4

(a)


COX-2
GAPDH
HA μg/cm²
0
.18
1.8
18
100
180
LB
As across
847 bp
550 bp

(b)

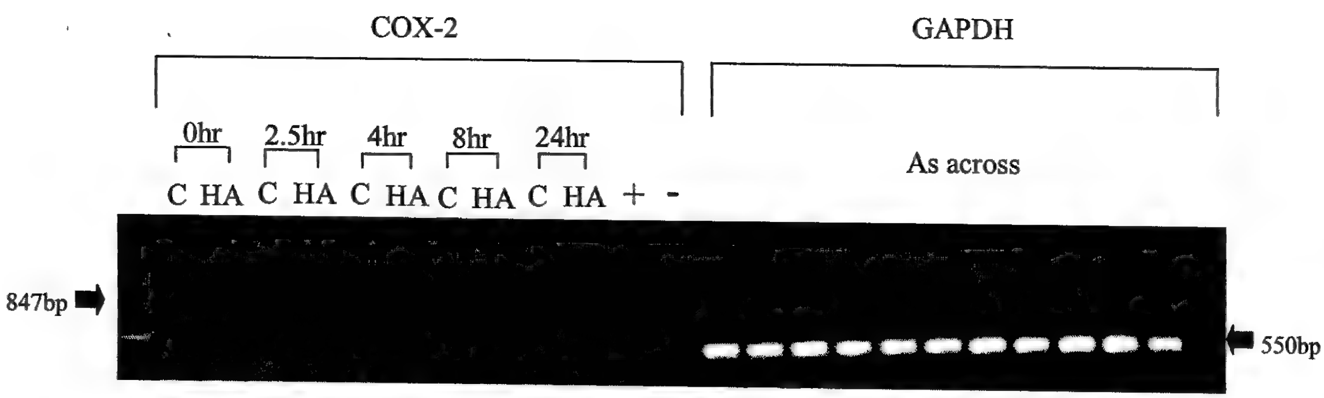

COX-2
GAPDH
0hr
2.5hr
4hr
8hr
24hr
C HA C HA C HA C HA C HA + -
As across
847bp
550bp

Fig 5

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Primary research

Inflammatory microcrystals induce murine macrophage survival and DNA synthesis

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Abstract

The interaction of particulates with resident macrophages is a consistent feature in certain forms of crystal-induced inflammation, for example, in synovial tissues, lung, and the peritoneum. The mitogenic activity of basic calcium phosphate (BCP) crystals and calcium pyrophosphate dihydrate (CPPD) crystals on synovial fibroblasts has been considered relevant to the synovial hyperplasia observed in crystal-induced arthritis. The aim of the study was to determine whether microcrystals such as these could enhance macrophage survival and induce DNA synthesis, thus indicating that they may contribute to the tissue hyperplasia.

Murine bone-marrow-derived macrophages were treated *in vitro* with microcrystals, the cell numbers were monitored over time, and DNA synthesis was measured as the incorporation of [methyl-^{3}H]thymidine (TdR). We report here that BCP, monosodium urate, talc, and, to a lesser extent, CPPD crystals promote macrophage survival and DNA synthesis; the latter response is particularly striking in the presence of low concentrations of macrophage-colony stimulating factor (M-CSF, CSF-1). Enhanced macrophage survival or proliferation may contribute to the synovial hyperplasia noted in crystal-associated arthropathies, as well as to talc-induced inflammation and granuloma formation. The crystals studied join the list of particulates having these effects on macrophages, indicating the generality of this type of response.

Keywords: crystals, DNA synthesis, macrophages, survival

Introduction

Intra-articular basic calcium phosphate (BCP) (hydroxyapatite, octacalcium phosphate, tricalcium phosphate) crystal-deposition disease is associated clinically with severe degenerative arthritis characterized by hyperplasia of the synovial lining and loss of intrasynovial collagenous structures [1]. The interaction of the crystals with inflammatory cells is believed to be a key factor in crystal-induced inflammation [2].

Deposition of calcium pyrophosphate dihydrate (CPPD) crystals has been associated with the acute inflammatory arthritis of 'pseudogout' [3]. Synovial thickening is a common clinical finding in the affected joints of patients with deposition of CPPD crystals. Histologically, synovial-cell hyperplasia and infiltration by mononuclear inflammatory cells are seen [3,4]; CPPD crystals are phagocytosed by cells of the synovial lining [4]. Gout, pseudogout, and acute articular and periarticular inflammation caused by

BCP = basic calcium phosphate; BMM = bone-marrow-derived macrophage; CSF-1 = colony stimulating factor-1; CPPD = calcium pyrophosphate dihydrate; FBS = fetal bovine serum; M-CSF = macrophage-colony stimulating factor; MSU = monosodium urate; oxLDL = oxidized low-density lipoprotein; RPMI = Roswell Park Memorial Institute [medium]; TdR = thymidine.

BCP crystals can behave similarly, reflecting the fact that BCP and CPPD crystals share many of the fundamental mechanisms of cell activation utilized by urate crystals [5,6]. Acute gout, even though initiated by deposition of monosodium urate (MSU) crystals in the synovium, is often associated with systemic inflammatory manifestations [7].

Talc (magnesium silicate) crystals, present in aerosols of respirable talc and surgical gloves, can cause, respectively, inflammatory reactions in the lung or a granulomatous reaction with peritoneal adhesions [8,9]. Exposure to talc leads to the development of granulomas containing macrophages [10]. Injection of talc into rabbit knees induces synovitis [11].

Macrophage-colony-stimulating factor (M-CSF), also called colony stimulating factor-1 (CSF-1), is an important regulator of the development and function of macrophage lineages throughout the body [12]. There are *in vivo* data suggesting that both synovial and peritoneal macrophages are completely dependent upon CSF-1 for their development [13].

One cell type that is normally resident in the synovium, and interacts *in vivo* with the various crystals mentioned above, is the macrophage (mononuclear phagocyte) [4]. We have previously shown that a number of particulates, including oxidized low-density lipoprotein (oxLDL) [14], adjuvants [15], and β-amyloid and prion protein fibrillogenic peptides (manuscript in preparation), were able to induce macrophage survival; they could also induce a proliferative response, particularly in the presence of circulating concentrations of CSF-1. We report here that BCP, MSU, talc, and, to a lesser extent, CPPD all promote the survival of murine bone marrow-derived macrophages (BMMs) and the synthesis of DNA, the latter response being potentiated again by low (suboptimal) CSF-1 concentrations.

Materials and methods

Bone marrow-derived macrophages, cell numbers, and DNA synthesis

As before, BMMs were generated as adherent cells from their nonadherent progenitors in the bone marrow of CBA mice (obtained from Monash University Animal Services, Clayton) and grown to confluence in 24-well plates (Nunc, Roskilde, Denmark) in the presence of CSF-1 (≥10,000 U/ml) [14,15]; the cells were prepared for an experiment by being washed twice with phosphate-buffered saline solution and the experiment was commenced immediately. For counting of BMMs, the medium was removed and the cells were gently scraped; viable cells were counted in a hemocytometer with the use of trypan blue exclusion [14]. DNA synthesis was measured as the incorporation of [methyl-^{3}H]thymidine (TdR) (2 μCi/ml) as before [14,15].

Crystals

BCP crystals were synthesized by a modification of published methods [6]. The crystals were crushed and sieved to yield aggregates10–20 μm in diameter, which were sterilized and rendered pyrogen free by heating at 200°C for 90 min. Triclinic CPPD crystals (10–50 μm in diameter) were synthesized and characterized as described elsewhere [16]. MSU monohydrate crystals (2–30 μm in diameter) were prepared from twice-recrystallized uric acid (Sigma Co, St Louis, MO, USA) and sodium hydroxide as previously described [17] and heated to 180°C for 2 h to render them pyrogen-free. Talc was US Pharmacopcia grade, as used clinically for pleurodesis; it was a gift from A Allison, Palo Alto, CA, USA. All crystals were resuspended in RPMI before culture.

Reagents

The following reagents were obtained from commercial sources: [^{3}H]TdR (80 Ci/mmol; Amersham Corp) and FBS (CSL, Parkville, Australia). Recombinant human CSF-1 (M-CSF) was a gift from Chiron Corp., Emeryville, CA, USA. All practical precautions for minimizing endotoxin contamination were taken [14,15].

Statistical analysis

Student's two-tailed *t*-test was used for the comparison of two mean values; $P < 0.05$ was considered statistically significant.

Results

Effect of crystals on macrophage survival

Upon removal of CSF-1, BMMs gradually die by apoptosis [18]. However, as seen in Table 1, BCP crystals and, to a lesser extent, CPPD crystals prevented cell loss. For BCP, there was still activity at 50 μg/ml, and the optimal concentration for enhanced survival was ≥100 μg/ml (data not shown). The concentrations of BCP and CPPD used were determined from previous studies of dose–response relationships and the mitogenic response in fibroblasts [17], and from the concentrations found in pathologic joint fluids [6].

Effect of crystals on macrophage DNA synthesis

We next determined whether the crystals induced BMMs to synthesize DNA. BCP stimulated BMM DNA synthesis in a dose-dependent manner (Fig. 1a); higher concentrations than those shown were toxic. CPPD, at its optimal concentration (500 μg/ml), stimulated BMM DNA synthesis (Table 2), albeit weakly in comparison with the effect of BCP and corresponding to its weaker effect on BMM survival.

MSU crystals promoted BMM DNA synthesis at the optimal concentration of 500 μg/ml (Table 3); they were active at ≥100 μg/ml (data not shown). The dose–response curve for talc, another biologically relevant particle, is presented in Fig. 1b; concentrations higher than those shown were toxic. The promotion of DNA synthe-

Table 1

Effect of BCP and CPPD on macrophage survival

Addition	No. of cells ($\times 10^{-4}$)
None	2.0±0.2
BCP	12.7±1.1
CPPD	4.7±0.2[a]

BMMs ($13.0 \pm 0.6 \times 10^4$) from which CSF-1 had just been removed were cultured in RPMI/10% FBS, in the absence or presence of BCP (500 μg/ml) or CPPD (500 μg/ml). After one day, cells were washed twice to remove cystals and recultured in RPMI/10% FBS for a further two days. Then the number of viable cells was determined by hemocytometer counting and trypan blue exclusion. Data are mean values ± SEM from triplicate cultures and are from a representative experiment that was repeated once. [a]$P < 0.05$ in comparison with untreated group (Student's two-tailed *t*-test).

Table 2

Effect of BCP and CPPD on macrophage DNA synthesis

Addition	[^{3}H]TdR incorporation (cpm $\times 10^{-4}$)
None	1.3±0.1
BCP	25.2±0.6
CPPD	3.1±0.1[a]

BMMs from which CSF-1 had just been removed were cultured as described in Table 1. After one day, cells were washed twice to remove cystals and recultured in RPMI/10% FBS containing [^{3}H]TdR (2 μCi) for a further two days. Data are mean values ± SEM from triplicate cultures and are from a representative experiment that was repeated eight times. [a]$P < 0.05$ in comparison with untreated group (Student's two-tailed *t*-test).

sis by MSU and talc indicates that they also prevented cell death.

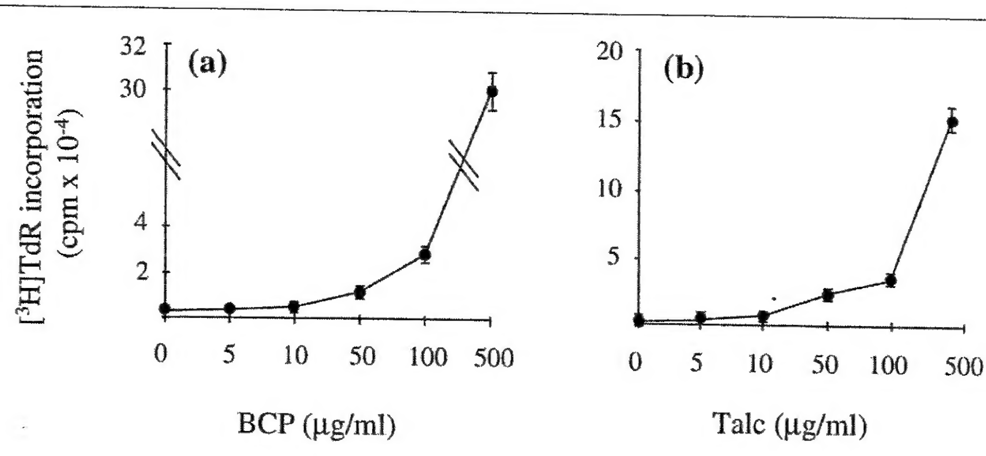
Effect of crystals in the presence of CSF-1

We have reported previously that other particulates, such as oxLDL and adjuvants, stimulate BMM DNA synthesis and they do this in a synergistic manner with CSF-1 [14,15]. As can be seen in Fig. 2, the stimulation of BMM DNA synthesis by BCP was potentiated by concentrations of CSF-1 that by themselves were suboptimal for the induction of DNA synthesis. For example, in the experiment whose data are provided, the [^{3}H]TdR incorporation value of $8.1 \pm 1.0 \times 10^4$ cpm resulting from the action of BCP alone rose to $22.4 \pm 3.1 \times 10^4$ cpm in the presence of 315 U/ml CSF-1, which by itself gave a value of $4.4 \pm 0.1 \times 10^4$ cpm. BMMs treated with MSU and talc also behaved similarly in the presence of a suboptimal concentration of CSF-1 (data not shown).

Discussion

We have shown that a range of biologically relevant crystals promote macrophage survival and DNA synthesis, the latter response being more dramatic in the presence of suboptimal concentrations of CSF-1. Such enhancement of macrophage survival by crystals, if it occurred *in vivo*, would lengthen the cells' tenure in a lesion (e.g. synovium), with the result that more cells would be present to produce inflammatory mediators, such as cytokines, proteinases, eicosanoids etc, perhaps potentiated by additional stimuli. This phenomenon could be another

Figure 1



Effect of BCP and talc doses on DNA synthesis in BMMs. BMMs from which CSF-1 had just been removed were cultured in RPMI/10%FBS, in the absence or presence of increasing concentrations of **(a)** BCP and **(b)** talc. After one day, cells were washed twice to remove crystals and recultured in RPMI/10%FBS containing [^{3}H]TdR for a further two days. Data are mean values ± SEM from triplicate cultures and are from a representative experiment that was repeated five times.

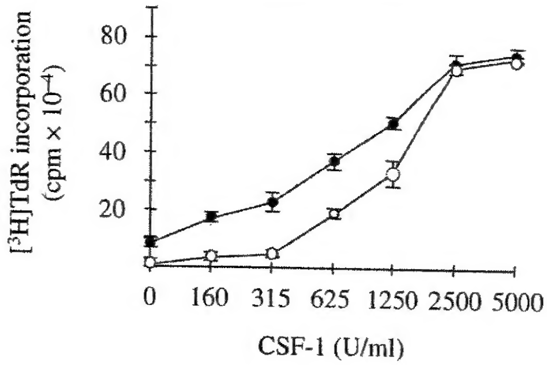
Table 3

Effect of monosodium urate on macrophage DNA synthesis

Addition	[^{3}H]TdR incorporation (cpm × 10^{-4})
None	1.6 ± 0.1
MSU	13.5 ± 0.5
BCP	24.3 ± 0.3

BMMs from which CSF-1 had just been removed were cultured in RPMI/10%FBS, in the absence or presence of monosodium urate (MSU) (500 μg/ml) or BCP (500 μg/ml). After one day, cells were pulsed with [^{3}H]TdR for a further two days. Data are mean values ± SEM from triplicate cultures and are from a representative experiment that was repeated twice.

Figure 2



Effect of BCP on DNA synthesis in BMMs in the presence of CSF-1. BMMs from which CSF-1 had just been removed were cultured in RPMI/10%FBS in the presence of increasing concentrations of CSF-1, in either the absence (○) or the presence (●) of BCP (100 μg/ml). After one day, cells were washed twice to remove BCP, recultured again in the increasing CSF-1 concentrations, and pulsed with [^{3}H]TdR for a further two days. Data are mean values ± SEM from triplicate cultures and are from a representative experiment that was repeated seven times.

mechanism contributing to the synovial hyperplasia attributed to crystals in joints [4]; it also may form part of how macrophage cell numbers increase in talc-induced granulomas and inflammatory reactions [10]. The degree of inflammation provoked experimentally by crystals *in vivo* is quite variable [5]. The capacity of crystal-treated monocytes or macrophages to produce inflammatory cytokines *in vitro* is likewise variable. For example, Malawista and coworkers found that the capacity of BCP and CPPD crystals to directly induce interleukin-1β and tumor necrosis factor-α in monocytes *in vitro* is weaker and less consistent than that of urate crystals [19]. In other words, the triggering of crystal-induced inflammation is not an 'all-or-nothing' response related to the new appearance of free crystals [5], and additional stimuli may be important.

The BMMs used in the above studies are a CSF-1-dependent population. As mentioned, there is evidence in the mouse that synovial and peritoneal macrophages are two populations that show CSF-1 dependence for their development [13]. In this sense, BMMs may represent a suitable model for these cells. It is likely that macrophages *in vivo* will be exposed to the low circulating concentrations of CSF-1, presumably responsible for their survival and steady-state turnover [12]. Since there is evidence of elevated CSF-1 concentrations in synovial fluids from patients with rheumatoid arthritis [20], those concentrations may also be raised in crystal-containing joints as part of the inflammatory reaction. CSF-1 can have proinflammatory effects in murine models of arthritis [21,22], and CSF-1-deficient (op/op) mice do not develop collagen-induced arthritis [22]. Our findings suggest that when macrophages are exposed to, or 'loaded' with, the range of crystals analyzed above, they are 'primed', so that they are able to proliferate better in the presence of CSF-1 doses that are suboptimal, i.e. that normally provide a survival signal or a weak proliferative one *in vivo*. If this potentiation were occurring *in vivo* with crystal-'loaded' macrophages, then again it could be contributing to the previously mentioned hyperplasia in joints and granulomas.

As discussed earlier, a number of clinical observations have suggested that articular inflammatory cells are 'primed' by crystals to heightened responsiveness to local stimuli [5]. Other observations have suggested that crystal-induced synovial fibroblast proliferation is a mechanism for increasing the number of synovial cells, which would then be capable of eliciting inflammatory mediators [17]. By analogy, our studies suggest that enhanced local macrophage survival or proliferation in the synovium (or in granulomas) should also be considered, in the same way, as a possible contributing factor. In support of this concept, there is evidence in a lentivirus model of arthritis that macrophages proliferate in the synovium [23] and in various types of granuloma [24].

The range of active materials promoting macrophage survival and DNA synthesis in the studies mentioned above indicates that calcium is not a necessary component. In synovial fibroblasts, by contrast, MSU only very slightly stimulated DNA synthesis, if at all, compared with BCP and CPPD [17]. However, in agreement with the synovial fibroblast response [17], latex did not support DNA synthesis in BMMs [14].

Crystals therefore join the list of particulates – for example, oxLDL [14] and certain adjuvants (aluminum salts, emulsions of oil in water, silica) [15] – that we have found to be capable of promoting macrophage survival and DNA synthesis, with potentiation again being observed with suboptimal CSF-1 concentrations. In each instance, including

that of the materials studied here, the responses of the macrophages have been proposed to account, in part at least, for the increased numbers of cells of macrophage lineage at various sites of chronic inflammation, for example, an atherosclerotic plaque, in response to particulates which are usually poorly degraded. Perhaps such a mechanism may be quite a general one, offering an explanation for the persistence (chronicity) of a number of inflammatory lesions leading to pathology. The molecular events responsible for the enhanced survival and proliferation await analysis.

Conclusion

BCP, MSU, talc, and, to a lesser extent, CPPD all promote murine macrophage survival and DNA synthesis, the latter response being potentiated by low (suboptimal) CSF-1 concentrations. Such enhanced macrophage survival or proliferation may contribute to the synovial hyperplasia noted in crystal-associated arthropathies, as well as to talc-induced inflammation. The crystals mentioned above can therefore be included in the list of particulates having these effects on macrophages, indicating the generality of this type of response.

Acknowledgements

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